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RNase III Participates in GadY-Dependent Cleavage of the *gadX-gadW* mRNA

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The adjacent *gadX* and *gadW* genes encode transcription regulators that are part of a complex regulatory circuit controlling the *Escherichia coli* response to acid stress. We previously showed that the small RNA GadY positively regulates *gadX* mRNA levels. The *gadY* gene is located directly downstream of the *gadX* coding sequence on the opposite strand of the chromosome. We now report that *gadX* is transcribed in an operon with *gadW*, although this full-length mRNA does not accumulate. Base pairing of the GadY small RNA with the intergenic region of the *gadX-gadW* mRNA results in directed processing events within the region of complementarity. The resulting two halves of the cleaved mRNA accumulate to much higher levels than the unprocessed mRNA. We examined the ribonucleases required for this processing, and found that multiple enzymes are involved in the GadY-directed cleavage including the double-strand RNA-specific endoribonuclease RNase III.

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Introduction

Regulatory RNAs in organisms from all kingdoms act by a wide array of mechanisms that enable them to play major roles in controlling gene expression. In bacteria, these regulatory RNAs are typically small in size (50–300 nt) and therefore have been termed small RNAs (sRNAs). Much of what is currently known about sRNA function in bacteria has arisen from studies of the Gram-negative model organism *Escherichia coli* in which around 80 sRNAs have been identified.[1] While not all of these sRNAs have been characterized, great strides have been made in understanding their mechanisms of action (reviewed in Ref. 2). The largest class of sRNAs elicits regulatory effects through the formation of base-pairing interactions with target mRNAs. Many of these sRNAs are encoded in *trans* with respect to their target mRNAs and contain only limited complementarity with the targets. In these cases, the RNA binding protein Hfq is usually required for sRNA function. Approximately one third of all *Escherichia coli* sRNAs bind to Hfq, and it is thought that each of these sRNAs acts by base pairing with *trans*-encoded mRNA targets. Four major regulatory outcomes have been established for the Hfq-dependent RNAs: inhibition or activation of translation or increased or decreased degradation of target mRNAs.

A limited number of base-pairing sRNAs that are encoded on the strand opposite annotated genes on bacterial chromosomes, and thus in *cis* to their

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Abbreviations used: sRNA, small RNA; UTR, untranslated region; RACE, rapid amplification of cDNA ends.

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targets, have also been characterized.[3] Due to this genetic arrangement, the sRNA and the target mRNA have the potential for extensive base pairing across the region of overlap. In these cases, the Hfq protein generally has not been found to be required for base pairing. Most of the *cis*-encoded sRNAs with known functions negatively regulate translation and promote the degradation of the complementary mRNA.

We have been characterizing the 105-nt *E. coli* GadY RNA, which is encoded in *cis* to the 3′ end of the *gadX* gene in the ~371-nt intergenic region between *gadX* and *gadW* (Fig. 1a). Intriguingly, this sRNA was found to positively regulate *gadX* mRNA levels.[4] The *gadX* mRNA encodes a transcription regulator that is involved in a highly complex regulatory circuit controlling the response to acid stress.[5,6] The *gadW* gene located immediately downstream from *gadX* also encodes a regulator of the acid response and can be transcribed with *gadX* or from its own promoter as an independent transcript.[5–8] We confirm here that *gadX* is transcribed as a two-gene mRNA with *gadW*. When the GadY sRNA is present, the *gadX-gadW* mRNA is processed to give rise to products with ends within the region complementary to GadY. Part of this processing is due to the GadY-dependent RNase III cleavage. However, GadY pairing with the *gadX-gadW* mRNA also modulates cleavage by other ribonucleases in mutants lacking RNase III.

Results

Region of complementarity confers GadY-dependent regulation on a heterologous gene

We previously showed that the GadY RNA positively regulates *gadX*, and that this regulation requires sequences in the 3′ untranslated region (UTR) of the *gadX* mRNA[4] (see also Fig. 1b). The 3′-UTR sequences of *gadX* needed for regulation are perfectly complementary to GadY due to the *cis*-encoded nature of these two RNAs, and it was proposed that base pairing within this region is necessary for regulation to occur.

We further investigated the mechanism of regulation by testing if the GadY RNA was capable of regulating a heterologous gene tagged with the 3′ UTR of *gadX*. A hybrid gene was constructed by replacing the *gadX* coding sequence with the coding sequence of the chloramphenicol resistance gene *cat*, such that the sequences downstream of the *gadX* coding sequence will be expressed as part of the *cat* mRNA (Fig. 1c). In addition, the *gadX* promoter was replaced with the *cat* promoter to eliminate the wild-type transcriptional regulation. To abolish the expression of the chromosomally encoded GadY RNA, the *gadY* promoter mutation described previously (TATATT -10 sequence replaced by GGGGGG) was also introduced into the strain. We then examined the levels of *cat* mRNA without and with GadY overexpression from a plasmid (Fig. 1d). The Northern blots showed that the *gadX* 3′ UTR is sufficient to confer GadY-dependent regulation on the heterologous *cat* gene. The strain carrying the pRI control vector had low levels of a band corresponding to the expected ~0.8 kb size of the *cat* mRNA. Multiple faint bands corresponding to longer transcripts that hybridized with the *cat* gene probe were also observed for this control strain. In contrast, only the ~0.8-kb *cat* mRNA was detected in the strain overexpressing GadY, and this transcript was present at much higher levels.

GadY RNA directs processing

The longer *cat* transcripts observed in the absence of GadY suggested that transcription of the reporter was proceeding through *cat* to downstream sequences. In the presence of the GadY RNA, these possible read-through transcripts were no longer detected. To confirm the read-through transcription, we made an additional modification to the *cat* reporter construct. A promoterless *gfp* gene encoding the green fluorescent protein was inserted into the chromosome downstream from *cat* and separated from the *cat* gene by the sequences complementary to GadY such that the native promoters for *gadW* and *gadY* were eliminated. The 3′ UTR of *gadX*, encompassing the region of base pairing with GadY, remained intact. The *gfp* gene was also engineered to contain *rrn* terminators downstream from the coding sequence so that any transcripts reading through *gfp* would be forced to terminate (Fig. 1e). We assayed for *gfp* expression in the absence or presence of GadY by examining cells under a fluorescent microscope. Unexpectedly, the reporter strain was fluorescent under both conditions, indicating that transcription initiating at the *cat* promoter was proceeding through *gfp* regardless of GadY expression (data not shown). We then examined each gene in the reporter operon by Northern blot analysis in strains carrying the control vector or overexpressing GadY. When GadY was absent, a single hybridizing mRNA was detected when the Northern blots were probed with the *cat* specific probe (Fig. 1f). The size of this mRNA (~1.7 kb) was consistent with a two-gene transcript encompassing both *cat* and *gfp* (Fig. 1e). When GadY was overexpressed, a single hybridizing mRNA was detected, but now the size of the transcript (~0.8 kb) was consistent with a single gene mRNA encompassing only the *cat* gene (Fig. 1e). Similar results were seen when the same total RNA samples were probed with a *gfp*-specific oligonucleotide. In the absence of the