The mating response of Enterococcus faecalis cells carrying the conjugative plasmid pCF10 is controlled by multiple regulatory circuits. Initiation of transcription of the prgQ conjugation operon is controlled by the peptide receptor protein PrgX; binding of the pheromone peptide cCF10 to PrgX abolishes PrgX repression, while binding of the inhibitor peptide iCF10 enhances repression. The results of molecular analysis of prgQ transcripts and genetic studies suggested that the elongation of prgQ transcripts past a putative terminator (IRS1) may be controlled by the interaction of nascent prgQ mRNAs with a small antisense RNA (Anti-Q) encoded within prgQ. Direct evidence for interaction of these RNAs, as well as the resulting effects on readthrough of prgQ transcription, has been limited. Here we report the results of experiments that (i) determine the inherent termination properties of prgQ transcripts in the absence of Anti-Q; (ii) determine the direct effects of the interaction of Anti-Q with nascent prgQ transcripts in the absence of complicating effects of the PrgX protein; and (iii) begin to dissect the structural components involved in these interactions. The results confirm the existence of alternative terminating and antiterminating forms of nascent prgQ transcripts in vivo and demonstrate that the interaction of Anti-Q with these transcripts leads to termination via inhibition of antiterminator formation. In vitro transcription assays support the major results of the in vivo studies. The data support a model for Anti-Q function suggested from recent studies of these RNAs and their interactions in vitro (S. Shokeen, C. M. Johnson, T. J. Greenfield, D. A. Manias, G. M. Dunny, and K. E. Weaver, submitted for publication).

The Enterococcus faecalis tetracycline resistance plasmid pCF10 is a member of a family of novel mobile genetic elements whose conjugative transfer functions are regulated by cell-cell communication (14). The E. faecalis chromosome encodes a peptide pheromone, cCF10 (LVTLVFV), that is released into the growth medium by plasmid-free recipient cells and serves as an indicator of recipient cell population density for donor cells carrying pCF10. In addition to a set of genes encoding functions necessary for conjugative mating pair formation and DNA transfer (13), pCF10 encodes a cCF10 sensing system, as well as two gene products, PrgY and iCF10 (inhibitor peptide; AITLIFI), that prevent donor cells from self-induction of conjugation by endogenous pheromone (11, 18). The iCF10 peptide functions as a competitive inhibitor of cCF10 (25). The intracellular target of both peptides is PrgX, a regulatory protein that serves as the molecular switch controlling transcription initiation of the prgQ operon, which encodes the pCF10 conjugation machinery (3, 6, 22, 29). iCF10 acts as a corepressor with PrgX and enhances the formation of a PrgX tetramer complex that favors repression of the prgQ promoter, P0 (22). Binding of cCF10 to PrgX has the opposite effect and leads to induction (29). Interestingly, iCF10, whose production is required to maintain repression in the absence of recipient cells, is encoded by a small open reading frame (ORF) within the first 86 nucleotides (nt) of prgQ transcripts (25) (Fig. 1C). Therefore, uninduced donor cells must express this ORF but not the downstream genes.

Molecular and genetic analysis of prgQ transcription revealed that uninduced cells carrying pCF10 produce a constitutive transcript, Qs (380 nt) (Fig. 1A) from the prgQ promoter, P0. The 3′ end of Qs corresponds to an inverted repeat sequence followed by a polyuridine tract, both of which suggest that IRS1 is a factor-independent terminator (1, 35). Pheromone-induced cells produce increased levels of Qs, as well as longer transcripts that extend >20 kb through the conjugation genes but are subjected to extensive processing, yielding stable products such as QL, an RNA with the same 5′ end as Qs but extended 130 nt at the 3′ end (6). Pheromone induction thus causes quantitative as well as qualitative changes in the prgQ transcripts that are produced, and pheromone-sensitive repression of P0 by PrgX explains the former but not the latter.

A previous study identified several cis-acting mutations in the prgQ region that cause an increase in transcription past IRS1 (4). Analysis of these mutations and of prgQ and prgX expression (2) led to the discovery that both strands of DNA in

Direct Evidence for Control of the Pheromone-Inducible prgQ Operon of Enterococcus faecalis Plasmid pCF10 by a Countertranscript-Driven Attenuation Mechanism†‡

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the prgQ locus are actively transcribed. We identified the constitutive prgX promoter, PX, about 220 bp from the prgQ transcription start site (but on the opposite strand and oriented in the opposite direction) (Fig. 1C). A 102-nt stable small RNA molecule, Anti-Q, is derived from the 5’/H11032 terminus of transcripts from PX, whereas the 3’/H11032 end of the same transcript encodes PrgX (2). Several of the cis-acting mutations (4) mapped to regions that were predicted to code for the helices of stem-loop structures within both Anti-Q and nascent prgQ transcripts. This suggested that the structure of these RNAs is an important factor in regulating transcription past IRS1.

Based on the resemblance of IRS1 to a factor-independent terminator, the complementarity of transcripts from both strands of the prgQ locus, the predicted secondary structures of Anti-Q and Qs, and analysis of the cis-acting mutants (4), it was proposed that nascent Qs could form two alternative secondary structures, one favoring termination at IRS1 and the other favoring antitermination (Fig. 1A; also see Fig S4 in the supplemental material). Interaction between Anti-Q and nascent prgQ transcripts was predicted to enhance formation of the terminator, and relatively low expression of prgQ transcripts in uninduced cells would ensure that virtually all of this RNA would be paired with Anti-Q, resulting in efficient termination and low downstream gene expression (Fig. 1B). Upon induction, the increased synthesis of prgQ transcripts would result in titration of all of the Anti-Q, and the remaining unpaired nascent prgQ transcripts would form the antiterminator structure, leading to extension of transcription into the downstream genes (4). This model is similar to the countertranscript-driven attenuation mechanisms that regulate copy number in plasmids of other Gram-positive bacteria (8), such as pT181 (26), pAM/H92521 (23), and pIP501 (10). However, in the case of pCF10, the countertranscript Anti-Q is derived from the 5’ end of an mRNA that also encodes PrgX, the repressor of PQ. Since antisense RNAs similar to Anti-Q are also encoded by other pheromone-responsive plasmids (30; S. Shokeen, C. M. Johnson, T. J. Greenfield, D. A. Manias, G. M. Dunny, and K. E. Weaver, submitted for publication), this proposed mechanism is likely to be of sufficient importance to be conserved through a significant period of plasmid evolution (21).

Although Anti-Q-mediated termination fits well with the available data and makes biological sense, there is a limited...
amount of direct evidence to support this model. The overlapping coding regions of the two transcripts introduce inherent complications in distinguishing direct effects of RNA/RNA interactions from those resulting from multiple effects of PrgX on transcripts produced from both promoters (3). We recently determined the secondary structure of Anti-Q and demonstrated direct interaction of Qs and Anti-Q in vitro (Shokeen et al., submitted). The results confirmed the structure of Anti-Q predicted from sequence analysis and provided important insights into the kinetics of complex formation and the subdomains of the two RNAs that mediate interactions. These studies identified a subdomain of Anti-Q containing a YUNR “U-turn” loop (8, 15). Studies of the countertranscript-driven attenuation system regulating copy number control of pIP501 demonstrated direct interaction of Qs and Anti-Q from pIP501 (2) using primers which appended the terminator downstream of Anti-Q, preventing transcription of extraneous sequences from PX. The net result of this cloning strategy was the removal of PQ and placement of the terminator downstream of Anti-Q. This fragment was digested with BamHI and AvrII and cloned into a similarly digested pXCAT backbone (2), resulting in pQaCAT. The net result of this strategy was the removal of PQ and placement of the terminator downstream of Anti-Q, preventing transcription of extraneous sequences from PX.

In this report, we describe the development of a genetic system that allows us to examine the inherent termination activity of unpaired nascent prgQ transcripts in vivo and the direct effects of Anti-Q on transcription readthrough of IRS1. This in vivo system allowed us to study the RNA/RNA interactions in the absence of other regulatory components involved in the pheromone response. Additionally, we present the results of in vitro transcription assays that provide evidence for the function of IRS1 as a terminator and a role for Anti-Q in favoring termination. Our results generally confirm those obtained by Shokeen et al. (submitted) and also provide strong evidence that interactions between Anti-Q and nascent prgQ transcripts directly affect readthrough at IRS1.

MATERIALS AND METHODS

**Bacterial strains, plasmids, and reagents.** Escherichia coli DH5α was used to propagate the plasmids in this study and was grown in LB broth or 0.075% yeast extract and 0.08% nutrient broth (YENB) (Difco) (28) or on LB plates with 1.5% agarose. Antibiotics at the following concentrations were used for E. coli: chloramphenicol, 20 μg/ml, and erythromycin, 200 μg/ml. E. faecalis OG15p, a spectinomycin-resistant derivative of strain OG1, was used for all experiments and was grown in Todd-Hewitt broth or M9-YE and glucose broth or on Todd-Hewitt plates with 1.5% agarose. Antibiotics at the following concentrations were used for *E. faecalis*: spectinomycin, 1,000 μg/ml; chloramphenicol, 20 μg/ml; and erythromycin, 10 μg/ml.

**Plasmid construction.** The plasmids used in this study are shown in Table 1; all DNA oligonucleotides used in this study are given in Table S1 in the supplemental material. pBK1 was constructed by excising the Xhol-BamHI fragment from pBK2 (Shokeen et al., submitted) and inserting it into a similarly digested pC13340 backbone (17). This plasmid has the prgQ transcript inserted into IRS1 but lacks prgX. pDM4 was constructed as follows. The Anti-Q region was amplified from pQCAT (2) using primers which appended the prgX terminator downstream of Anti-Q. This fragment was digested with BamHI and AvrII and cloned into a similarly digested pXCAT backbone (2), resulting in pQCAT. The net result of this cloning strategy was the removal of PQ and placement of the prgX terminator downstream of Anti-Q, preventing transcription of extraneous sequences from PX.

**Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Nucleotides</th>
<th>Relevant features</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBK1-1</td>
<td>pBK1 carrying the antiterminator deletion (Δ120–253)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pBK1-25</td>
<td>pBK1 derivative carrying complementary mutations to 3 nt in each stem half of the terminator: prgQ</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pBK1-13</td>
<td>pBK1 derivative with lacZ transcriptionally fused to prgQ +354</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pBK1-14</td>
<td>Derivative of pBK1-13 also carrying the antiterminator deletion (Δ120–253)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pBK1-17</td>
<td>pBK1 derivative carrying a deletion between IRS1 and the lacZ junction (Δ385–394)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pBK1-18</td>
<td>pBK1 derivative carrying a deletion of the 3’ half of IRS1 (Δ354–376)</td>
<td>This study</td>
<td></td>
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<tr>
<td>pBK1-19</td>
<td>Derivative of pBK1-18 also carrying the antiterminator deletion (Δ120–253)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>pBK1-21</td>
<td>pBK1 derivative carrying complementary mutations to 3 nt in each stem half of the terminator as well as the compensatory mutation within the antiterminator: prgQ 339–41GGG→CCC, 369–71CCC→GGG (Fig. 1C)</td>
<td>This study</td>
<td></td>
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<tr>
<td>pBK1-24</td>
<td>pBK1 derivative carrying a mutation in Qs complementary to the U in the Anti-Q YUNR loop (A215G)</td>
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<td>pBK1-25</td>
<td>pBK1 derivative carrying a deletion that inactivates PX (A228C)</td>
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<td>pAMβ1-based, Gram-positive E. coli shuttle vector</td>
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<td>pDM4</td>
<td>pAT18 derivative containing prgQ +1 through +390, produces Anti-Q</td>
<td>This study</td>
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<tr>
<td>pDM4-3</td>
<td>pDM4 derivative carrying a point mutation within the proposed YUNR loop of Anti-Q (T9C)</td>
<td>This study</td>
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Quantitative reverse transcription-PCR (qRT-PCR). *E. faecalis* strains were grown overnight in M9-YE broth at 37°C with selective antibiotics. In the morning, cultures were diluted 1:5 in fresh medium and grown for 90 min at 37°C. Then, 600 µl of each culture was added to 1,200 µl of RNAProtect bacteria reagent (Qiagen), prepared according to the reagent instructions, snap-frozen in ethanol and dry ice, and stored at −80°C. RNA was prepared using an RNeasy kit (Qiagen) following a modified enzymatic lysis procedure (5). RNA (2 to 5 µg) was treated with Turbo DNase (Ambion) according to the manufacturer’s instructions. RNA (20 ng) was then reverse transcribed using a Superscript III first-strand synthesis kit (Invitrogen) and gene-specific primers (GSPs), following the manufacturer’s instructions. Each reaction mixture contained GSPs for lacZ and gvrB. Approximately 1 ng cDNA was then used for quantitative PCR using IQ SYBR green Supermix (Bio-Rad) on an IQ5 real-time PCR system (Bio-Rad). The amplification efficiency of each primer set was assessed by amplifying 10-fold serial dilutions of a DNA control consisting of linearized pBK1 (lacZ) or *E. faecalis* genomic DNA (gvrB). Transcript levels were then determined relative to the level of pBK1 by using differences in the threshold cycle (ΔC\textsubscript{T} method), with gvrB serving as the reference gene.

RNA stability assay. *E. faecalis* strains were cultured as described above for the qRT-PCR assays. After the 90-min subculture, rifampin (350 µg/ml) was added to 5 ml of each culture. At time zero and 5, 10, 20, 40, and 80 min, 600-µl aliquots were removed from each culture. RNA was prepared from these samples as described for the qRT-PCR assay. RNA (1 µg) from each sample was electro- phoresed on a 1.2% denaturing agarose gel in MOPS (morpholinepropanesulfonic acid) buffer as previously described (2). RNA was transferred to a positively charged nylon membrane (Roche) by passive elution with 5 M NaOH for 2 h (http://www.ambion.com/techlib/tb/169.html). Membranes were cross-linked using a Stratalinker (Stratagene).

RNA probes were generated as follows. Primers were designed to amplify the region of interest and append an SP6 promoter to one end of the PCR product such that transcription produced a probe complementary to the region of interest. The PCR product was then used as a template for SP6 RNA polymerase (RNAP) to generate a digoxigenin-UTP (DIG-UTP; Roche)-labeled RNA probe according to the manufacturer’s instructions.

Membranes were probed with the DIG-labeled RNA probes according to the manufacturer’s instructions, and probe hybridization was detected using DIG-specific Fab fragments and CDP-star reagent (Roche). Approximately 1 ng cDNA was then used for quantitative PCR using IQ SYBR green Supermix (Bio-Rad) on an IQ5 real-time PCR system (Bio-Rad). The amplification efficiency of each primer set was assessed by amplifying 10-fold serial dilutions of a DNA control consisting of linearized pBK1 (lacZ) or *E. faecalis* genomic DNA (gvrB). Transcript levels were then determined relative to the level of pBK1 by using differences in the threshold cycle (ΔC\textsubscript{T} method), with gvrB serving as the reference gene.

RESULTS

Evidence for competing terminator/antiterminator structures within nascent *prgQ* transcripts. In order to investigate the activities of the proposed terminator and antiterminator structures, we cloned the pCF10 region from *P*\textsubscript{Q} to IRS1 into a reporter plasmid with a *lacZ* transcriptional fusion downstream of the terminator (pBK1) (Fig. 1). This allowed us to use β-galactosidase expression and *lacZ* transcript abundance to report extension of transcripts past IRS1. Although pBK1 retains *P*\textsubscript{X}, it lacks the *prgX* structural gene and produces very little Anti-Q, consistent with previous results (2). Therefore, we used pBK1 to examine the intrinsic termination behavior of nascent *prgQ* transcripts and mutant derivatives without the influence of these negative regulators. Initially, we used β-galactosidase assays to assess transcription past IRS1 in pBK1 and its derivatives (see Fig. S1 and Table S2 in the supplemental material). We then used qRT-PCR to quantify *lacZ* mRNA levels directly.

IRS1 contains a G+C-rich inverted repeat that can form a stable stem-loop structure, followed by a uridine-rich region [poly(U) tract], both characteristic of factor-independent transcriptional terminators (1, 35). We hypothesized that disrupting IRS1 would lead to a decrease in termination, which would result in an increase in transcription past IRS1. To test this, we compared the *lacZ* transcript abundance of pBK1 and a pBK1-derived construct with a deletion of the 3’ side of the inverted repeat (pBK1-18) (Fig. 2), predicted to prevent formation of the terminator stem. This mutation had no effect on *lacZ* transcript levels, suggesting that IRS1 does not act as an intrinsic terminator in the context of our plasmid.

These results conflicted with previous interpretations of Northern blots and sequence data suggesting that IRS1 is a factor-independent terminator. To reconcile these results, we hypothesized that, in the absence of Anti-Q RNA, formation of a previously postulated antiterminator (4) is constitutive within nascent *prgQ* transcripts, precluding terminator formation and allowing extension into downstream sequences.

To test this hypothesis, we deleted the 5’ side of the antiterminator (pBK1-1) (Fig. 2C). We reasoned that in the absence of the competing antiterminator, the terminator should form readily. *lacZ* transcript abundance from a reporter carrying this mutation dropped to 12% of that from pBK1 (AT deletion, Δ120–253) (Fig. 2B), supporting the model that the antiterminator precludes termination at IRS1.

If disruption of the antiterminator decreases *lacZ* transcript abundance by allowing IRS1 to act as a terminator, then deletion of IRS1 should suppress the effect of this disruption. To test this, we introduced the antiterminator deletion into pBK1-18, which carries a deletion of the 3’ side of IRS1. This construct displayed no change in *lacZ* transcript abundance relative to that of pBK1-18 (Fig. 2). Taken together, these data indicate that the antiterminator deletion decreases *lacZ* transcript abundance by allowing formation of the terminator.

To further examine the ability of IRS1 to terminate transcription, we swapped 3 nt between the 5’ and 3’ sides of the IRS1 helix. This mutation (pBK1-4) maintained the structure of the terminator but, because the 5’ side of IRS1 participates in formation of the antiterminator, it was predicted to destabilize the antiterminator element. This mutation reduced *lacZ* transcript abundance to 16% of that of pBK1 (Fig. 2), consistent with reduced antitermination. To ensure that this change in expression was due to the loss of complementarity within the antiterminator, we constructed plasmid pBK1-21, which is identical to pBK1-4 except that it carries an additional change in the 5’ side of the antiterminator that restores complemen-
tarity. This construct exhibited an increase in lacZ transcript abundance of 290% compared to that of pBK1-4 (67% compared to pBK1), partially suppressing the phenotype displayed by pBK1-4. These data support our model in which IRS1 can act as a terminator, but this is precluded by the formation of an upstream antiterminator within the nascent RNA.

**Anti-Q promotes termination at IRS1.** We next wanted to examine the direct effects of Anti-Q on nascent prgQ transcript termination at IRS1 in the absence of any potential complicating factors, such as PrgX control of PrG. Despite the presence of the Anti-Q promoter and the Anti-Q sequence, wild-type cells carrying pBK1 produce very little Anti-Q. It has previously been demonstrated that in cells containing these sequences, PrgX protein must be provided in trans for Anti-Q to be expressed at appreciable levels (4). In contrast, pDM4 contains a smaller segment of pCF10 DNA that includes the P trans promoter and DNA encoding Anti-Q but not PrG. Cells containing pDM4 produce abundant levels of Anti-Q in the absence of PrgX, consistent with previous results (3), allowing us to provide Anti-Q in trans.

Using qRT-PCR, we found that providing Anti-Q in trans to pBK1 reduced lacZ transcript abundance to 28% of its abundance from pBK1 without Anti-Q (Table 2). This supports the argument that Anti-Q acts on nascent prgQ transcripts to cause terminator formation. The reporter bearing a deletion of the 3′ side of IRS1 was not sensitive to Anti-Q (pBK1-18) (Table 2), demonstrating that IRS1 is necessary for Anti-Q to decrease the expression of downstream genes. Taken together, these data support the model that Anti-Q interacts with nascent prgQ transcripts to allow termination at IRS1.

We also analyzed the impact of Anti-Q on prgQ transcript termination when the antiterminator was destabilized by point mutations within the terminator (pBK1-4) (Table 2). Because the antiterminator was already destabilized, reducing lacZ transcript abundance to 16% compared to pBK1, we expected that Anti-Q would have only a modest ability to further disrupt the antiterminator, making this mutant less sensitive to Anti-Q than the wild type. Surprisingly, Anti-Q reduced lacZ transcript abundance from pBK1-4 to 10% of the level with pBK1-4 alone, a statistically significant difference (P = 0.01) from the response of pBK1 to Anti-Q, indicating that this mutant is more responsive to Anti-Q. Providing the compensatory mutation within the antiterminator appeared to partially alleviate this increased sensitivity to Anti-Q, as Anti-Q reduced lacZ transcript abundance produced by this construct to 16% of the level with the construct alone, though this change in response was not statistically significant (pBK1-21) (Table 2).

An alternate explanation for some of the above-described results could be that mutations within prgQ or interaction between nascent prgQ transcripts and Anti-Q cause rapid degradation of the fused lacZ transcript. In order to test this, we examined the half-life of lacZ transcripts from different strains during log-phase growth by halting transcription with rifampin and examining the rate of transcript decay, as detailed in Materials and Methods.
The effect of mutating the U residue in the YUNR motif of Anti-Q in pDM4 (pDM4-3). As a control, we also introduced the complementary mutation into pBK1 (pBK1-24). The results in Fig. 3 show that this point mutation did not significantly affect basal lacZ transcript abundance from the reporter alone (pBK1 versus pBK1-24). We used pDM4 and pDM4-3 to provide either wild-type or mutated Anti-Q in trans to both pBK1 and pBK1-24 and measured lacZ transcript levels in each of these strains. The strains with wild-type prgQ (pBK1) showed no difference in sensitivity to wild-type or mutated Anti-Q. Similarly, the strains carrying a mutation complementary to the YUNR mutation were equally sensitive to both wild-type and mutated Anti-Q. These results confirm that this YUNR loop does not play a physiological role in Anti-Q-mediated termination at IRS1, consistent with previous results (Shokeen et al., submitted).

**Termination and antitermination function in vitro.** In order to confirm our interpretations of the in vivo study results, we examined termination and antitermination using runoff (IVT) assays. We found that, in the absence of Anti-Q, 41% of nascent prgQ transcripts terminated at IRS1 (Fig. 4A and B). When unlabeled Anti-Q RNA was added to the IVT reaction, 88% of nascent prgQ transcripts terminated at IRS1, a significant increase ($P = 0.01$). The addition of a control RNA similar in size to Anti-Q did not alter the frequency of termination at IRS1. Taken together, these data support the model that nascent prgQ transcripts contain a transcriptional terminator at IRS1, and Anti-Q interaction with these transcripts supports termination.
Previously work on pCF10 revealed that control of the extension of prgQ transcripts past IRS1 is an important checkpoint in the pheromone response (6). In uninduced conditions, extended transcripts are not detectable by Northern blot, whereas they become readily detectable after pheromone induction. Analysis of predicted secondary structures of Qs RNA revealed that IRS1 resembles an intrinsic terminator of transcription but that upstream sequences may form a competing antiterminator structure capable of precluding IRS1 formation in nascent transcripts (see Fig S4 in the supplemental material). A small RNA, Anti-Q, is transcribed from the DNA strand opposite prgQ. Since Anti-Q is complementary to a portion of the antiterminator, interactions between Anti-Q and nascent prgQ transcripts could disrupt the antiterminator, thus allowing terminator formation (4).

Studies of countertranscript-driven attenuation mechanisms are complicated by the fact that both the regulatory RNA and the target RNA are produced from the same region of DNA. A prior study of prgQ regulation identified cis-acting mutations in the region of complementarity between prgQ and Anti-Q that affected the expression of genes downstream of IRS1 (4). A number of these mutations mapped to predicted stem structures within Anti-Q or prgQ. Because DNA in this region is transcribed on both the regulatory and target RNAs, we frequently could not determine which RNA was impacted by a particular mutation. We were also unable to identify regions of the RNAs that must be complementary for optimal interaction, since any mutations cause complementary changes in both RNAs. In the current study, we developed a system for producing Anti-Q and prgQ transcripts from separate plasmids in vivo. We provided prgQ on a plasmid that produces very little Anti-Q and reports transcript extension past IRS1. We provided Anti-Q in trans on a compatible plasmid that lacks PCO. This arrangement allowed us to directly test the effect of Anti-Q on termination at IRS1. It also allowed us to introduce mutations into one transcript at a time and to test their effects on either Anti-Q or prgQ transcripts or both RNAs simultaneously.

The results in Fig. 2 and Table 2 demonstrate that, in vivo, IRS1 acts to terminate nascent prgQ transcripts but that, in the absence of Anti-Q, this activity is suppressed by an upstream antiterminator. The addition of Anti-Q allows for termination at IRS1. These results are supported by the results of IVT assays that demonstrate that prgQ transcripts can terminate at or extend past IRS1 and that the addition of Anti-Q RNA increases the frequency of termination, probably by direct interaction with nascent prgQ transcripts.

A mode of direct interaction between Anti-Q and nascent prgQ transcripts is supported by (i) the success of Anti-Q in enhancing termination in vitro when RNAIP is the only protein factor present and (ii) the results of in vitro studies that demonstrate that Qs and Anti-Q can interact in the absence of additional factors (Shokeen et al., submitted). This is consistent with results from in vitro studies of other RNA/RNA interactions in which the regulatory and target RNAs are encoded in the same region of DNA and share high complementarity (9, 16). This is different than many well-studied RNA/RNA systems in which the regulatory RNA is encoded in a different genomic location than the target and the two share limited complementarity. In these trans-coded systems, a host protein factor is needed for productive interactions between the regulatory RNA and the target. In eukaryotes, Argonauta proteins support interaction between mature microRNAs and their targets (12), and in bacteria, trans-coded RNAs typically require Hfq for productive interactions with their targets (32). E. faecalis does not have an identified Hfq homolog. It is possible, however, that an unidentified host factor is needed for optimal interactions between these cis-coded RNAs in vivo. It is interesting that our in vivo results suggest that termination does not occur at IRS1 in the absence of Anti-Q, while our in vitro results demonstrate termination in the absence of Anti-Q. This could be due to differences in the biochemical conditions within the cell compared to the IVT reaction. It may also be interpreted as supporting the existence of a host factor that mediates optimal RNA/RNA interaction in vivo but which is absent from the IVT assays.

Recently, Shokeen et al. (submitted) determined the secondary structure of Anti-Q in vitro and characterized its interactions with Qs. We found that Anti-Q contains three stem-loop structures, including one with a YUNR motif. YUNR motifs have been shown to accelerate cognate RNA/RNA interactions (15) and play an important role in other countertranscript-driven attenuation systems (19). Surprisingly, the loop within Anti-Q containing the YUNR motif was not protected by interaction with Qs. Additionally, a mutation that disrupted the YUNR loop had little effect on extension past IRS1 when introduced on a plasmid in vivo. This plasmid produced both prgQ transcripts and Anti-Q in cis, so the mutation was transcribed on both RNAs. From these results it was clear that the YUNR motif was not critical for Anti-Q-driven attenuation at IRS1; however, it was uncertain if disrupting the complementarity of the YUNR loop between Anti-Q and nascent prgQ transcripts would have any effect. By introducing the mutation to prgQ and Anti-Q separately, we have demonstrated that a loss of complementarity within this loop does not affect the ability of Anti-Q to enhance termination at IRS1. This agrees with the results of in vitro experiments (Shokeen et al., submitted) in which disruption of the YUNR loop in Anti-Q had no effect on the rate or extent of interaction between Anti-Q and wild-type Qs.

When prgQ is transcribed, the region complementary to the YUNR loop is transcribed after the regions complementary to the other two loops of Anti-Q, making it the last region available for interaction. However, when Anti-Q is transcribed, the YUNR loop is the first loop structure expressed. It is possible that, given its position in Anti-Q, the YUNR loop serves to accelerate interactions between mature Qs and nascent transcripts from PX. This could serve as a regulatory mechanism for the expression of prgX, the distal gene transcribed from PX. If such regulation exists, it would be a novel example of reciprocal regulation of distal transcripts between regulatory RNAs transcribed from two loci; this possibility is being tested experimentally.

When nucleotides were exchanged between the stems of IRS1, destabilizing the antiterminator (pBK1-4) (Fig. 2; Table 2), the reporter showed the expected loss of downstream gene expression. Contrary to expectations, this reporter was more responsive to Anti-Q than pBK1. We hypothesize that, by
disrupting the antiterminator, this mutation stabilizes an up-
stream region within nascent prgQ transcripts that interacts with Anti-Q. The results of Shokeen et al. (submitted) indicate that only certain portions of Anti-Q are protected through interaction with prgQ transcripts. We interpret this to mean that the interactions between Anti-Q and nascent prgQ tran-
scripts occur between specific motifs of the two molecules rather than along the entire region of complementarity. Future work will focus on identifying and characterizing this upstream region within prgQ.

pBK1-1 contains a deletion of the antiterminator. Since prgQ transcripts from pBK1-1 do not possess a structure to compete with terminator formation, terminal formation should be at maximum efficiency. pBK1-4 contains an exchange of 3 nt between the stems of IRSI. When Anti-Q is added in trans to pBK1-4, it expresses less lacZ than pBK1-1. The reasons for this are unclear, but it is possible that the mutated IRSI may be a more efficient terminator or that antiterminator disruption enhances Anti-Q binding to nascent prgQ transcripts.

Control of the transfer function in pCF10 is a complex pro-
cess regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ. This is regulated by a variety of mechanisms, many of which converge to control the fate of transcripts initiated at PQ.

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REFERENCES


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