Identification of a conserved branched RNA structure that functions as a factor-independent terminator
Identification of a conserved branched RNA structure that functions as a factor-independent terminator

Christopher M. Johnson*,1,2, Yuqing Chen*,1, Heejin Leeb, Ailong Keb, Keith E. Weaveera, and Gary M. Dunny*a,3

*Department of Microbiology, University of Minnesota, Minneapolis, MN 55455; †Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853; and ‡Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD 57069

Edited by Susan Gottesman, National Institutes of Health, Bethesda, MD, and approved January 21, 2014 (received for review August 13, 2013)

Anti-Q is a small RNA encoded on pCF10, an antibiotic resistance plasmid of Enterococcus faecalis, which negatively regulates conjugation of the plasmid. In this study we sought to understand how Anti-Q is generated relative to larger transcripts of the same operon. We found that Anti-Q folds into a branched structure that functions as a factor-independent terminator. In vitro and in vivo, termination is dependent on the integrity of this structure as well as the presence of a 3′ polyuridine tract, but is not dependent on other downstream sequences. In vitro, terminated transcripts are released from RNA polymerase after synthesis. In vivo, a mutant with reduced termination efficiency demonstrated loss of tight control of conjugation function. A search of bacterial genomes revealed the presence of sequences that encode Anti-Q-like RNA structures. In vitro and in vivo experiments demonstrated that one of these functions as a terminator. This work reveals a previously unappreciated flexibility in the structure of factor-independent terminators and identifies a mechanism for generation of functional small RNAs; it should also inform annotation of bacterial sequence features, such as terminators, functional sRNAs, and operons.

A n essential function of all cells is to terminate transcription elongation at the 3′ ends of genes or operons to prevent aberrant expression of adjacent loci encoding unrelated functions. In bacteria, virtually all known termination systems use one of two general mechanisms. In factor-dependent terminations, events such as release of ribosomes from nascent transcripts or DNA damage can stimulate termination at distal locations lacking a specific sequence signature; protein factors, such as Rho and Mfd, distinct from the core RNA polymerase complex, play an essential role in dissociation of the transcription elongation complexes. In contrast, factor-independent or intrinsic termination is effected by a specific RNA structure in the nascent transcript comprised of a stable GC-rich hairpin or “hyphenated inverted repeat,” often followed by a run of U residues (polyuridine tract) (1–3). This latter form of termination can occur in vitro with core RNA polymerase (RNAP) in the absence of additional proteins, although accessory proteins, such as NusA, can enhance the process substantially (1). Additional terminator geometries, which include a terminal hairpin structure, have been proposed (4). Additionally, some bacteria have been shown to encode terminators that lack a polyuridine tract (5, 6). Despite these exceptions, the factor-independent terminators studied to date show remarkable conservation of the canonical hairpin/polyuridine structure, and this feature is the essential function of all cells is to terminate transcription elongation at the 3′ ends of genes or operons to prevent aberrant expression of adjacent loci encoding unrelated functions. In bacteria, virtually all known termination systems use one of two general mechanisms. In factor-dependent terminations, events such as release of ribosomes from nascent transcripts or DNA damage can stimulate termination at distal locations lacking a specific sequence signature; protein factors, such as Rho and Mfd, distinct from the core RNA polymerase complex, play an essential role in dissociation of the transcription elongation complexes. In contrast, factor-independent or intrinsic termination is effected by a specific RNA structure in the nascent transcript comprised of a stable GC-rich hairpin or “hyphenated inverted repeat,” often followed by a run of U residues (polyuridine tract) (1–3). This latter form of termination can occur in vitro with core RNA polymerase (RNAP) in the absence of additional proteins, although accessory proteins, such as NusA, can enhance the process substantially (1). Additional terminator geometries, which include a terminal hairpin structure, have been proposed (4). Additionally, some bacteria have been shown to encode terminators that lack a polyuridine tract (5, 6). Despite these exceptions, the factor-independent terminators studied to date show remarkable conservation of the canonical hairpin/polyuridine structure, and this feature is the primary sequence signature used to predict the 3′ termini of transcriptional units in bacterial genomes (7–11).

In the pCF10 system, regulatory interactions between the pheromone-responsive gene (prg) Q conjugation operon and prgX repression operon allow cells with pCF10 to detect the presence of potential recipients in a sensitive, yet tightly controlled fashion, and transfer pCF10 to those recipients via conjugation (see refs. 12 and 13 for reviews). The 5′ segments of both operons encode stable regulatory small RNAs (sRNA), and proteins are encoded downstream (Fig. 1). The prgX repression operon encodes the PrgX repressor protein, which represses transcription initiation at the prgQ promoter (PQ), thereby repressing conjugation (14). The 5′ terminal segment of the prgX operon also encodes Anti-Q (previously Qs), an sRNA which interacts with nascent transcripts of the prgQ conjugation operon to terminate transcription, attenuating expression of conjugation genes (15, 16). This report addresses the mechanisms by which Anti-Q is generated and how expression levels of the RNA and protein products of the prgQ and prgX operons are coordinated.

Anti-Q is present in donor cells as a distinct transcript from the prgX promoter. Previous work has shown that Anti-Q can be produced in the absence of PrgX protein and Qs. However, it was unclear if Anti-Q was produced by processing of longer transcripts or by termination (17). We report that Anti-Q functions as a factor-independent terminator with an unusual branched structure, followed by a polyuridine tract, and that these features are sufficient for termination. Termination occurs in vivo and in vitro, with terminated transcripts being released from RNAP in vitro. Mutational analysis suggests that the mechanism of termination is the similar to that of canonical intrinsic terminators. The termination efficiency of Anti-Q is lower than that of conventional enterococcal terminators, allowing cell to produce a mixture of short and long transcripts with distinct functions from the prgX promoter (PS) (Fig. 1). We found that reducing Anti-Q termination impairs the regulatory function of the Anti-Q sRNA, making cells sensitive to lower levels of pheromone. This finding suggests that the Anti-Q sRNA prevents conjugation in response to minor fluctuations in extracellular

Significance

All cells regulate where regions of DNA are transcribed to RNA. Controlling where transcription terminates is an essential part of this regulation. In bacterial cells, RNA structures, referred to as factor-independent terminators, can interact with RNA polymerase to direct termination. These structures are typically inverted sequence repeats that form an RNA hairpin followed by several uridine residues. We identified a branched RNA structure that functions as a factor-independent terminator. The terminated product is a functional small RNA, but termination is inefficient, allowing transcription of downstream genes. Additional branched terminators are encoded in bacterial chromosomes, demonstrating that this unusual terminator is not unique. This work reveals an unappreciated structural diversity of factor-independent terminators and will inform annotation of bacterial genomes.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

*C.M.J. and Y.C. contributed equally to this work.

†Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

‡To whom correspondence should be addressed. E-mail: dunny001@umn.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1315374111/-/DCSupplemental.
phero
domine or an +X mRNA targeted by RNase III is bracketed. The sequence of the Anti-Q/and the promoter at P +X +mRNA +are +shown +as +open +arrows +labeled +Q +and +X, +respectively. The PrgX primary and secondary binding sites are shown as double and single triangles, respectively. Wavy lines above and below the map indicate RNAs with transcription initiated at P +X +and +Anti-Q +region. DNA is shown at the top as a dark line with features indicated. Transcriptional start sites are shown as bent arrows; +RNAP. We used plasmids 

Johnson et al. to 5 +E. faecalis +with +different +amounts +of +downstream +sequences +as 

pro− Anti-Q +and +RNase III digestion and is likely a result of the increased sensitivity between these results and those obtained previously by S1 nuclease mapping (15, 111, 119). Arrows indicate the right terminus of various pCF10 fragments tested for Anti-Q formation, as described in the text (+107, +117, +223). Brackets indicate sequence removed by defined deletions. The proposed polyuridine tract is underlined. IRSX and IRS1 denote stem-loop structures that function in transcription termination.

Results

Sequences Within the 5’ Region of the prgX Repression Operon Are Sufficient to Generate Anti-Q RNA. Anti-Q is encoded at the 5’ terminus of the prgX repression operon. A strain carrying pDM4 (Table S1), which contains a segment of pCF10 from P −176 to +223, but does not include P +O or the prgX ORF (Fig. 1), produced abundant Anti-Q (15, 16, 18). The 3’ terminus of Anti-Q produced from this plasmid and from pCF10 was located at P + +107, as determined by a 3’ RACE protocol. The slight difference between these results and those obtained previously by S1 nuclease mapping (15) is likely a result of the increased sensitivity of 3’ RACE or a low level of 3’ to 5’ RNA degradation during the S1 procedure.

To identify sequences necessary to generate Anti-Q, we cloned pCF10 fragments carrying P + and different amounts of the 5’ end of the prgX operon into plasmids and used Northern blots to analyze Anti-Q production from E. faecalis carrying these plasmids. A plasmid carrying sequences to P +107 did not produce Anti-Q, but a plasmid that carrying sequences to P + +117 did (Fig. 2A). We previously determined that prgX/Qs RNA duplexes are subject to RNase III−dependent processing (17) in the region shown in Fig. 1. We tested whether this pathway could also result in generation of Anti-Q and found that an RNase III knockout strain actually had much higher levels of Anti-Q, ruling out this processing pathway for generation of Anti-Q in vivo (Fig. 2B).

Anti-Q Functions as a Factor-Independent Terminator. The sequence between P + +107 and P + +117 is thymidine-rich, resembling the polyuridine tract of factor-independent terminators. The structure of Anti-Q has been examined by Pb** digestion and is a three-way branching structure rather than the hairpin characteristic of such terminators (19) (Fig. S1). However, Anti-Q functioned as a factor-independent terminator during in vitro transcription assays with E. faecalis RNAP. We used plasmids carrying P + with different amounts of downstream sequences as templates and found Anti-Q was generated if the polyuridine tract was present, but not if it was absent (Fig. 2C).

These Anti-Q transcripts were not associated with RNAP, indicating that they are not a result of a paused or stalled RNAP complexes. When transcription was performed bead-bound RNAP (20, 21), Anti-Q was depleted in the bead fraction and enriched in the supernatant, indicating that it is no longer associated with RNAP (Fig. 2D). Other bands were enriched in the bead fraction, demonstrating that the protocol did not cause nonspecific dissociation of the RNA from RNAP. We conclude that Anti-Q is generated as the result of transcription termination.

Anti-Q does not require that transcription initiate at P + to function as a terminator. We cloned Anti-Q into a plasmid downstream of the prgQ promoter at P + +96 +and +used +this +as +a +template +for +in +vitro +transcription +reactions. Anti-Q terminates transcription in this context (Fig. 3A, lane 1), indicating that intrinsic termination does not depend on transcription initiating at P +.

Fig. 1. A map of the P +P − region. DNA is shown at the top as a dark line with features indicated. Transcriptional start sites are shown as bent arrows; terminators are shown as stem−loops; the prgQ and prgX ORFs are shown as open arrows labeled Q and X, respectively. The PrgX primary and secondary binding sites are shown as double and single triangles, respectively. Wavy lines above and below the map indicate RNAs with transcription initiated at P + and P −, respectively. The region of the prgX mRNA targeted by RNase III is bracketed. The sequence of the Anti-Q/prgX sense strand is shown below. The two mapped transcriptional start sites are indicated (+1, +4). Open diamonds ( ) indicate the 3’ termini of Anti-Q in vivo as determined by S1 endonuclease mapping (+102) and 3’ RACE ( +107). Closed circles ( ) indicate in vitro termination points ( +111, +119). Arrows indicate the right terminus of various pCF10 fragments tested for Anti-Q formation, as described in the text (+107, +117, +223). Brackets indicate sequence removed by defined deletions. The proposed polyuridine tract is underlined. IRSX and IRS1 denote stem−loop structures that function in transcription termination.

Fig. 2. Anti-Q is generated by transcription termination. (A) Northern blot showing RNA harvested from OG1sp carrying plasmids with P + and Anti-Q sequences through +117 (pCJ15, lane 1) or +107 (pCJ14, lane 2) with an ectopic terminator fused downstream. The membrane was hybridized with a RNA probe specific for Anti-Q. E. faecalis 5S is shown as a loading control. (B) Northern blot of RNA harvested from either wild-type E. faecalis or an isogenic RNase III deletion mutant strain (OG1RF Δ3097) carrying pBK2 and probed for Anti-Q or 5S RNA as in A. (C) In vitro transcription reactions performed using the plasmids pCJ15 (lane 1) and pCJ14 (lane 2) as templates. (D) An in vitro transcription reaction performed using RNAP that was adsorbed to Nickel−agarose beads. The template was a linear PCR product containing P −167 to +400 with P + inactivated by a mutation to its −10 region. Lane 1, complete reaction; lane 2, RNAP associated products; lane 3, free products.
Anti-Q Terminates at Two Closely Spaced Residues. When wild-type P$_x$ sequences were truncated at +117, Anti-Q formed a single band (Figs. 2 A and C, 3A lane 1, and Fig. S2), but when downstream sequences were present, Anti-Q formed two bands during in vitro transcription reactions (Fig. 2D). Transcription from P$_x$ is initiated at two different positions, three nucleotides apart (17), possibly explaining the two bands. However, a plasmid (pCF19) in which P$_x$ drove transcription of Anti-Q also produced two bands. These bands were located within an extended polyuridine tract at U111 and U119, each immediately downstream of a G residue, as determined by a chain termination reaction (Fig. 3D and Fig. S3). The U119 termination point was not included in the template used for many of the experiments (Figs. 2 A and B, and 3A, and Fig. S2), explaining the differences between the experiments.

Integrity of the Anti-Q Structure Is Important for Termination. To understand sequence and structural determinants of Anti-Q termination, we identified randomly generated Anti-Q mutants that changed the frequency of termination during in vitro transcription assays. We sequenced and mapped these to the experimentally determined structure of Anti-Q (Fig. 3D and Fig. S2). Generally, we only considered templates that carried a single mutation. However, a template with both A18G and C97U showed a dramatic decrease in termination, so we cloned these mutations separately to dissect their individual contributions. None of the mutations identified increased the frequency of termination. Rather, all of them decreased Anti-Q termination frequency (Fig. 3C, black bars). The majority of mutations were located within the stems of Anti-Q, and none were located within the outside loops.

Some mutations fell outside the stems of the Anti-Q RNA secondary structure (A22G, A23G, A36G, U112C) (Fig. S1), which was determined previously using +102 as the 3’ terminus (19). Sequence analysis revealed that extending the 3’ terminus of Anti-Q to include the polyuridine tract might allow additional base-pairing, extending the basal stem to include A22 and A23 and stem II to include A36. Therefore, mutation of these residues may also affect Anti-Q secondary structure (Fig. 3D).

Mutations within the basal stem generally reduced termination the most (Fig. 3) (A22G, A23G, A33G, C97U). Mutations within stem I (U15C, A18G) had the least effect, whereas those within stem II (A36G, U40G, U44G) or stem III (U67C, A75C, A78G) reduced termination by varying degrees. The mutation U112C had the most dramatic effect on Anti-Q termination. This residue is part of the polyuridine tract, and likely affects termination by disrupting this feature. These results suggest that sequences within the stems of Anti-Q as well as the polyuridine tract are involved in transcription termination.

Mutations predicted to suppress stem-disrupting mutations increased termination frequency. We chose templates with stem-disrupting mutations and generated compensatory mutations to restore the structure of the disrupted stem. In five of the six cases tested, this significantly increased termination in vitro ($P < 0.02$) (Fig. 3C, white bars, and Table S2). Most of the alleles with a termination-reducing mutation had an increased AG, relative to wild-type, indicating Anti-Q was destabilized. The compensatory mutations increased terminator stability. However, in agreement with previous studies, the correlation between the predicted stability of Anti-Q and the frequency of termination was low (22, 23) (Fig. S4 and Table S2).

The Anti-Q terminator has structural features not identified by previous studies, as revealed by lead-acetate cleavage (Fig. S5). The stretch of nucleotides between the loop of stem I and the three-way junction is protected from cleavage, supporting the notion that the basal stem is extended in wild-type Anti-Q and includes residues A22, A23, and A36 (Fig. 3D). The region where the base of stem II meets the three-way junction is sensitive to cleavage, suggesting that stem II may not be extended. Importantly, there is a stretch of protected bases between the loop of stem III and the bulge in the basal stem, suggesting that stem III stacks on top of the basal stem. These results are generally consistent with the structure of Anti-Q shown in Fig. 3D.

Most mutations that decreased termination alter the Anti-Q structure. The C97U and A33G mutations destabilize the basal stem with A33G disrupting the stacking of stem III onto the basal stem (Fig. S5). With the A33G mutation, this result is inconsistent with the minimum free-energy predictions (Table S2).

---

Fig. 3. Disrupting Anti-Q structure lowers frequency of termination. (A) In vitro transcription reactions performed using plasmid templates that contained P$_x$ fused to Anti-Q with deletions to different regions of Anti-Q: lane 1, wild-type; lane 2, Δ34–65; lane 3, Δ66–95. The region in which wild-type and truncated Anti-Q sized products appear is indicated. The bands at the top of the gel are the loading wells. (B) RNA was isolated from strains carrying plasmids with P$_x$ driving different Anti-Q alleles: lane 1, wild-type Anti-Q; lane 2, a vector control; lane 3, Δ34–65; lane 4, Δ66–95. Blots were probed for Anti-Q (Upper) or SS5 (Lower). (C) In vitro transcription reactions using PCR products as templates that contained P$_x$ and Anti-Q sequences through +117 with an ectopic terminator (IRS1) downstream. The frequency of termination at Anti-Q was calculated by dividing by the intensity of the Anti-Q band by the intensity of the IRS1 band. Values were normalized to termination at Anti-Q in a reaction with the wild-type template. Bars are labeled with the mutations carried on each template. White bars indicate templates that carry mutations predicted to restore secondary structure. Asterisks indicate a significant difference in termination frequency ($P < 0.02$). (D) The proposed structure of Anti-Q as a functional terminator. The different stems are labeled. The in vitro termination points, as described in the text, are labeled with asterisks. Experimentally tested mutations are labeled. Regions removed by the stem deletions are boxed.
indicating the mutation may disrupt the 3D structure of the terminator in a way we have not resolved. Secondary structure predictions indicate that this mutation shifts the location of the three-way junction, shortening the basal stem and lengthening stem III by one nucleotide. The U104G and A75C mutations destabilize stems II and III, respectively. The A26G mutation, however, does not alter the structure appreciably. These results are generally consistent with a model in which mutations that decrease termination act by altering the Anti-Q secondary structure. However, additional sequence-specific effects cannot be ruled out.

**Stems II and III of Anti-Q Both Play Roles in Intrinsic Termination.** Because deletion of either stem II or stem III of Anti-Q would likely generate a single stem/loop structure more closely resembling a typical terminator, we tested the effects of these deletions in vitro and in vivo. A plasmid in which the stem II-coding region had been deleted (Δ34–65) (Fig. 3A, lane 2) produced an apparent termination product in vitro, whereas a plasmid in which stem III had been deleted (Δ66–95) (Fig. 3A, lane 3) did not. Using Northern blotting, we found that strains bearing a plasmid with either deletion mutant produced an RNA that hybridized to an Anti-Q probe (Fig. 3B). This finding suggests that either the requirements for termination are less stringent in vivo than in vitro or that Anti-Q-like RNAs can also be produced via processing of longer transcripts. It is noteworthy that deleting stem III—but not stem II—dramatically decreases Anti-Q stability (Table S2). To further characterize the apparent termination product produced by the stem II deletion mutant, we carried out an in vitro transcription experiment using a template carrying the stem II deletion and RNA polymerase attached to beads, similar to the experiment depicted in Fig. 2D. The RNA produced from this reaction was less abundant than observed with a wild-type template, but was detected in the supernatant fraction, suggesting that it was generated by termination (Fig. S6 A and B). However, when we analyzed the termination site, it appeared to be in a polyuridine tract (residues 96–103) that is part of the basal stem of wild-type Anti-Q (Fig. S6C). Thus, stem II may not be essential for termination per se, but enforces termination at the proper location, possibly by preventing backtracking of the paused polymerase through the basal stem region.

**Termination at Anti-Q Affects Expression of Genes in the prgX Repression Operon.** We wished to explore the role of Anti-Q termination in regulating gene expression from the prgQ conjugation and prgX repression operons. Manipulating the Anti-Q polyuridine tract reduced Anti-Q production in vivo (Fig. 4A). We generated mutations to the Anti-Q polyuridine tract and cloned these into pBK2, which carries the prgX repression operon, as well as the prgQ conjugation operon, through inverted repeat sequence 1 (IRS1) (Fig. 1 and Table S1) with a beta-galactosidase (lacZ) gene downstream of IRS1. We used Northern blots to examine the amount of Anti-Q produced by strains transformed with these plasmids. Compared with wild-type (Fig. 4A, lane 1), deleting the region containing the first termination site (Δ107–113) dramatically reduced Anti-Q production (Fig. 4A, lane 2). Deleting the second termination site (Δ116–120) or making single-point mutations to either of the terminal nucleotides (U112C, U119C) reduced Anti-Q production (Fig. 4A, lanes 3–5) to a lesser extent.

Anti-Q is part of a polycistronic operon, encoded upstream of the prgX gene. Reducing the efficiency of termination at Anti-Q might be expected to increase expression of prgX. Surprisingly, mutations which reduced Anti-Q expression had only minor effects on the abundance of unprocessed (1.4 kb) prgX mRNA and no significant effect on the abundance of the processed (1.2 kb) transcript detected by Northern blots (Fig. 4B). PrgX protein levels were reduced in both the Δ107–113 and U112C mutants (Fig. 4C) as determined by Western blotting. The lack of increase in 1.4 kb prgX abundance in the Δ107–113 and Δ116–120 mutants probably results from indirect effects of the mutations on the processing and stability of prgX transcripts, as further addressed in the Discussion.

**Termination at Anti-Q Affects Expression of Genes in the prgQ Conjugation Operon.** Anti-Q interacts with nascent prgQ transcripts, causing them to terminate at IRS1, producing Q$_\text{L}$ transcripts, as further indicated by Northern blotting (Fig. 4A). Q$_\text{L}$ production decreased in strains carrying three of the four mutant Anti-Q alleles, compared with the wild-type allele. Additionally, the Δ107–113 mutant showed a consistent, although statistically insignificant, increase in the amount of transcripts that extend past IRS1 (Q$_\text{L}$, 1.62 ± 0.32 compared with wild-type, $P = 0.07$, t test), suggesting a defect in Anti-Q driven attenuation. To further assess the role of the Anti-Q termination region on prgQ transcription, we used the lacZ gene fused downstream of IRS1 as a proxy for expression of conjugation genes. The Δ107–113 mutation caused an increase in β-galactosidase expression, whereas the other mutations did not (Fig. S7A). This finding is consistent with the possible increase in extended Q$_\text{L}$ transcript levels caused by the Δ107–113 mutation (Fig. 4A).

In the above experiments, the prgQ conjugation and prgX repression operons were oriented in their normal cis configuration and PrgX was present. Transcription interference between the operons impacts expression levels of RNAs and the PrgX repressor, modulating repression of the prgQ operon. Additionally, mutations to the sequence of Anti-Q also change the sequence of prgQ transcripts. These factors complicate interpretation of changes to prgQ transcript levels. To avoid these difficulties, we provided Anti-Q and transcripts from the prgQ conjugation operon from different plasmids using a previously described system that lacks the PrgX repressor (16). Under these conditions all mutations to the Anti-Q polyuridine tract affected prgQ transcript attenuation at IRS1, resulting in increased reporter expression (Fig. S7B). This result shows that Anti-Q produced from these alleles is less effective at attenuating expression of genes downstream of IRS1. Transcripts that extend past the normal Anti-Q
termination point may be less effective as attenuators of prgQ transcription or the mutations used to reduce termination after the native Anti-Q structure, impairing its function as an antisense RNA.

Changes to Anti-Q termination affected the pheromone induction response. Strains bearing pBK2 with or without the Δ107–113 mutation were induced with the pheromone cCF10 and prgQ transcription extension past the IRS1 attenuation checkpoint (Fig. 1) was monitored by qRT-PCR. When a low level of pheromone was added, the Δ107–113 mutant showed a low level of induction that was rapidly turned off, whereas the wild-type strain showed no induction (Fig. 5). When a high level of pheromone was added, both strains showed a rapid, high level of induction that tapered off. These data show that the strain with the mutant Anti-Q allele is sensitive to levels of pheromone that fall below the threshold needed to trigger induction of cells with wild-type Anti-Q.

Bacterial Chromosomes Encode Branched Terminators. The chromosomes of several Gram-positive bacteria appear to encode Anti-Q like terminators. The genomes of *E. faecalis* V583, *Lactococcus lactis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococcus pyogenes* all contained sequences that could encode branched structures followed by polyuridine tracts (Table S3). This result suggests that branched terminators may not be uncommon. At least one of the chromosomally encoded branched structures can function as a terminator. We selected five of the candidate sequences (Table S3), cloned them downstream of the P₀ promoter, and tested them by in vitro transcription reactions. The cloned t11562, sequence from *E. faecalis* directed formation of a pair of distinct bands, that were released from RNA polymerase bound to agarose beads, suggesting a terminated product (Fig. 6). The conditions for the in vitro transcription experiment had to be optimized to observe these bands, suggesting that the terminator functions at a low efficiency, consistent with our observations for Anti-Q. Other sequences that had Anti-Q–like characteristics did not direct termination. It is unclear if this is because the experimental conditions did not support such function, or if the sequences themselves are not terminators. The sequence for t11562 is located in an untranslated region upstream of the ORF of OG1RF_11562. A strain with a recombinant plasmid, pCY1790322, containing this sequence expressed an RNA of the correct size to be the terminated product, as determined by Northern blotting (Fig. 6C). The band is faint, suggesting either that termination is inefficient—consistent with the in vitro results—or that the product is unstable. We were unable to detect transcripts of any length containing this sequence in the absence of the plasmid, indicating that the chromosomal region does not produce abundant transcripts under the conditions tested.

**Discussion**

In this study we found that the sRNA, Anti-Q, is generated as a result of intrinsic transcription termination. The location at which Anti-Q terminates transcription in vitro is a few nucleotides downstream of the 3′ terminus of the mature Anti-Q RNA, suggesting that in vivo, Anti-Q undergoes posttranscriptional processing. Conceivably, Anti-Q–like RNAs could be generated from longer prgX transcripts via posttranscriptional processing. Genetic analysis of the effects of RNase III-dependent processing on Anti-Q levels (Fig. 2B) ruled out one obvious processing pathway. In addition, our cumulative results show aberrant regulation of the prgQ conjugation operon in all strains deficient in Anti-Q termination, indicating that the functional form of this RNA in wild-type cells is generated by termination.

Genes of the Anti-Q sRNA does not require other sequences or functions encoded by pCF10, including convergent transcription from the P₀ or Q₅ RNA, PrgX repressor protein, or transcription of sequences within the prgX repression operon downstream of the Anti-Q polyuridine tract, including the region of the prgX mRNA cleaved by RNase III (17). Additionally, Anti-Q termination does not require that transcription initiate at Pₓ. These data demonstrate that synthesis of Anti-Q is intrinsic to its coding sequence. Anti-Q shares some sequence and structural characteristics with canonical factor-independent terminators. Such terminators generally contain a simple G+C-rich stem loop, followed by a polyuridine tract, which may be unbroken, discontinuous, or absent (5, 6, 24). Anti-Q encodes a discontinuous polyuridine tract, mutation or deletion of which reduces or eliminates termination. In contrast, Anti-Q is a branched structure with a basal stem (Fig. 3, and Figs. S1 and S5). The branched structure is important for termination at the proper position. When either stem II or stem III sequences are removed, the predicted modified structure resembles the simple stem-loop of other terminators, although removal of either stem...
renders the RNA nonfunctional in attenuating prgQ transcription elongation (16, 19). This is a characteristic of systems in which a sRNA directs termination of a nascent transcript (25–27). Efficient contact with two loops of the sRNA (Anti-Q) is needed for attenuation, otherwise the interaction is too slow and the nascent transcript (Qs) extends past the possible termination point (28–30). Although deletion of stem III abolished termination in vitro (Fig. 3A), a stem II deletion retained partial termination function, but the termination site was altered (Fig. 3A and Fig. S6). Thus, stem II contributes to Anti-Q termination, likely facilitating stacking of stem III onto the basal stem. The biological constraint of maintaining both intrinsic termination and antisense RNA functions likely drove the evolution of the complex Anti-Q structure. As with other factor-independent terminators, disrupting base-pairing interactions within the stems reduced the frequency of termination. Disrupting the three-way junction between the stems also reduced termination.

The intrinsic termination function of Anti-Q impacts expression of other genes within the prgX repression and prgQ conjugation operons. Anti-Q is encoded within the 5′ region of the prgX mRNA and RNA termination cannot be too efficient, otherwise the downstream prgX ORF would not be expressed. Levels of prgX mRNA and PrgX protein were not directly coupled to the frequency of termination at Anti-Q. In wild-type donor cells there were two distinct antisense interactions between complementary RNAs produced from the prgQ and prgX promoters (16, 17). Kissing complexes between short, complementary loops of Anti-Q and nascent prgQ transcripts impact the secondary structure of the latter RNAs and control their termination at IRS1 (Fig. 1), whereas RNA duplexes between complementary unstructured regions of extended prgQ transcripts and 5′ prgQ mRNAs provide the initial substrates for RNase III-dependent processing of prgQ mRNA (17). The latter pathway requires prgX transcripts, but the former does not. In vivo, the increased production of extended prgQ transcripts in Anti-Q termination mutants may provide more prgX RNA for RNase III-dependent processing, resulting in no net alteration of the steady-state levels of prgX message.

A mutant Anti-Q allele impacted the induction of conjugation genes in response to the pCF10 pheromone. Cells with the mutant Anti-Q allele were induced at a low level by small amounts of cCF10, whereas cells with a wild-type Anti-Q allele were not. Given that the pheromone induction system of pCF10 exhibits properties of a bistable switch (18), Anti-Q could reduce the sensitivity of the system to noise, either minor fluctuations in external cCF10 levels or stochastic fluxes in prgQ transcription. This would prevent unproductive mating responses.

Annotation algorithms frequently identify terminators based on the presence of a stable G+C rich stem-loop followed by a polyuridine tract. Understanding structural variations on this theme that also terminate transcription will be useful in predicting operon structure and identifying additional functional sRNAs. A genome search identified several Anti-Q like structures in a variety of organisms. At least one of these structures is able to direct termination, showing that the branched Anti-Q terminator is not unique.

Materials and Methods

Methods are described in full in SI Materials and Methods. In vitro transcription assays were performed using E. faecalis RRNA and purified plasmid DNA as a template as described (20). To distinguish between terminated and nonterminated transcripts, the His-tagged E. faecalis RRNA was bound to nickel-agarose beads before the assay. Brief centrifugation was used to pellet the beads with associated RNAP and bound transcripts. Terminated transcripts were recovered from the supernatant. Random mutations to Anti-Q were introduced during PCR amplification by supplementing the buffer with 0.5 mM MnCl2 and excess dCTP. Randomly mutated sequences were ligated into an unmutated plasmid. The desired sequence with a wild-type promoter was then PCR-amplified from the plasmid to serve as a transcription template.

ACKNOWLEDGMENTS. We thank Alan Grossman for suggestions on the manuscript. This work was funded by Public Health Service Grant GM49530 (to G.M.D.).

9. Kashlev M, et al. (1996) Histidine-tagged RNA polymerase of E. coli and antisense RNA functions likely drove the evolution of the complex Anti-Q structure. As with other factor-independent terminators, disrupting base-pairing interactions within the stems reduced the frequency of termination. Disrupting the three-way junction between the stems also reduced termination.

The intrinsic termination function of Anti-Q impacts expression of other genes within the prgX repression and prgQ conjugation operons. Anti-Q is encoded within the 5′ region of the prgX mRNA and RNA termination cannot be too efficient, otherwise the downstream prgX ORF would not be expressed. Levels of prgX mRNA and PrgX protein were not directly coupled to the frequency of termination at Anti-Q. In wild-type donor cells there were two distinct antisense interactions between complementary RNAs produced from the prgQ and prgX promoters (16, 17). Kissing complexes between short, complementary loops of Anti-Q and nascent prgQ transcripts impact the secondary structure of the latter RNAs and control their termination at IRS1 (Fig. 1), whereas RNA duplexes between complementary unstructured regions of extended prgQ transcripts and 5′ prgQ mRNAs provide the initial substrates for RNase III-dependent processing of prgQ mRNA (17). The latter pathway requires prgX transcripts, but the former does not. In vivo, the increased production of extended prgQ transcripts in Anti-Q termination mutants may provide more prgX RNA for RNase III-dependent processing, resulting in no net alteration of the steady-state levels of prgX message.

A mutant Anti-Q allele impacted the induction of conjugation genes in response to the pCF10 pheromone. Cells with the mutant Anti-Q allele were induced at a low level by small amounts of cCF10, whereas cells with a wild-type Anti-Q allele were not. Given that the pheromone induction system of pCF10 exhibits properties of a bistable switch (18), Anti-Q could reduce the sensitivity of the system to noise, either minor fluctuations in external cCF10 levels or stochastic fluxes in prgQ transcription. This would prevent unproductive mating responses.

Annotation algorithms frequently identify terminators based on the presence of a stable G+C rich stem-loop followed by a polyuridine tract. Understanding structural variations on this theme that also terminate transcription will be useful in predicting operon structure and identifying additional functional sRNAs. A genome search identified several Anti-Q like structures in a variety of organisms. At least one of these structures is able to direct termination, showing that the branched Anti-Q terminator is not unique.

Materials and Methods

Methods are described in full in SI Materials and Methods. In vitro transcription assays were performed using E. faecalis RRNA and purified plasmid DNA as a template as described (20). To distinguish between terminated and nonterminated transcripts, the His-tagged E. faecalis RRNA was bound to nickel-agarose beads before the assay. Brief centrifugation was used to pellet the beads with associated RNAP and bound transcripts. Terminated transcripts were recovered from the supernatant. Random mutations to Anti-Q were introduced during PCR amplification by supplementing the buffer with 0.5 mM MnCl2 and excess dCTP. Randomly mutated sequences were ligated into an unmutated plasmid. The desired sequence with a wild-type promoter was then PCR-amplified from the plasmid to serve as a transcription template.

ACKNOWLEDGMENTS. We thank Alan Grossman for suggestions on the manuscript. This work was funded by Public Health Service Grant GM49530 (to G.M.D.).