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Transcriptional initiation of a small RNA, not R-loop stability, dictates the frequency of pilin antigenic variation in *Neisseria gonorrhoeae*

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Abstract

Neisseria gonorrhoeae, the sole causative agent of gonorrhea, constitutively undergoes diversification of the type IV pilus. Gene conversion occurs between one of several donor silent copies located in distinct loci and the recipient *pilE* gene, encoding the major pilin subunit of the pilus. A guanine quadruplex (G4) DNA structure and a cis-acting sRNA (G4-sRNA) are located upstream of the *pilE* gene and both are required for pilin antigenic variation (Av). We show that reduced sRNA transcription lowers pilin Av frequencies. Extended transcriptional elongation is not required for Av, since limiting the transcript to 32 nt allows for normal Av frequencies. Using chromatin immunoprecipitation assays (ChIP), we show that cellular G4s are less abundant when sRNA transcription is lower. In addition, using ChIP, we demonstrate that the G4-sRNA forms a stable RNA:DNA hybrid (R-loop) with its template strand. However, modulating R-loop levels by controlling RNase HI expression does not alter G4 abundance quantified through ChIP. Since pilin Av frequencies were not altered when modulating R-loop levels by controlling RNase HI expression, we conclude that transcription of the sRNA is necessary, but stable R-loops are not required to promote pilin Av.

Abbreviated Summary

Neisseria gonorrhoeae, the causative agent of gonorrhea, changes one of its surface exposed structures through DNA recombination, in a process known as antigenic variation. A small RNA is required for antigenic variation and we found that though it forms an RNA:DNA hybrid,hybrid stability is not important for antigenic variation. It is transcription of the small RNA that allows for the formation of an alternate DNA structure called a guanine quadruplex, that drives antigenic variation.

Graphical abstract

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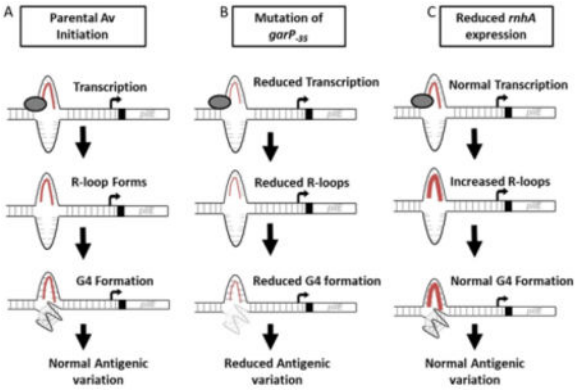
Author contributions

LLP and HSS designed the study. LLP, EAO, LAC, and HSS participated in the acquisition, analysis, or interpretation of the data. LLP and HSS wrote the manuscript.



Keywords

R-loop; guanine quadruplex; antigenic variation; recombination; gene diversification

Introduction

Programmed genetic recombination allows for diversification of genes in many forms of life. Various bacterial, eukaryotic and viral pathogens utilize gene diversification to allow for immune avoidance and functional modification(s) (Palmer *et al.*, 2016, Deitsch *et al.*, 2009, Holland *et al.*, 1982). These organisms alter surface structures through phase variation of genes, error prone replication, altered partitioning of genomic segments, and homologous recombination. In addition, mammalian immune cells use programmed genetic recombination to allow diversity in B cell responses (Maizels, 2005). Understanding how these species undergo high rates of recombination or mutation, while preserving genome function and viability over many generations is yet to be understood.

Neisseria gonorrhoeae (Gc) is a strict human pathogen and the causative agent of the sexually transmitted infection, gonorrhea (Quillin & Seifert, 2018). Symptomatic Gc infection is characterized by a robust innate immune response consisting of an influx of polymorphonuclear leukocytes to the site of infection. However, Gc can survive in and around these innate immune cells and possesses multiple mechanisms to avoid adaptive immune recognition, including antigenic variation (Av). The Type IV pilus of Gc and the closely-related pathogen *Neisseria meningitidis* (Mc), is an essential virulence factor. The Gc pilus promotes host cell adherence, biofilm formation, twitching motility, DNA transformation, and resistance to polymorphonuclear leukocytes (Rudel *et al.*, 1992, Sparling, 1966, Wolfgang *et al.*, 1998, Park *et al.*, 2001, Stohl *et al.*, 2013). Gc and Mc vary the gene encoding the major pilin subunit of the pilus, *pilE*, through changes in the coding sequence (Hagblom *et al.*, 1985). Throughout the Gc and Mc genomes, distinct loci encode variant copies of the major pilin subunit that lack a promoter sequence, ribosome binding site, and signal sequence, known as silent copies (*pilS*). During pilin Av, a portion of a silent copy sequence replaces the analogous part of the *pilE* gene in a gene conversion process (Haas & Meyer, 1986, Meyer *et al.*, 1984, Swanson *et al.*, 1987). Any portion of the donating silent copy can be transferred into the recipient *pilE* locus, resulting in an altered *pilE* gene and thereby altering the pilin protein sequence. After gene conversion, the silent

copy gene sequence, within its original locus, remains unchanged. Pilin Av requires many conserved recombination and repair factors that normally function in single strand gap and double strand break repair including RecA, RecOR, and RecG (Sechman *et al.*, 2005, Mehr & Seifert, 1998, Mehr & Seifert, 1997). When other recombination and repair factors, RecQ, RecJ, and Rep, are mutated, pilin Av is lowered, but not totally abrogated, indicating these factors are involved in the gene conversion process, but may have redundant roles (Mehr & Seifert, 1998, Skaar *et al.*, 2002, Kline & Seifert, 2005, Rotman *et al.*, 2016, Xu & Seifert, 2018).

A conserved sequence located upstream of *pilE* can form an alternate DNA structure called a guanine quadruplex (G4) (Cahoon & Seifert, 2011, Cahoon & Seifert, 2009). G4 structures have been suggested to play specific roles in many molecular processes, including telomere maintenance, transcriptional regulation, DNA replication, viral packaging, and recombination (Rhodes & Lipps, 2015, Maizels & Gray, 2013). While historically the physical properties of G4 structures have been studied extensively *in vitro*, understanding the biological functions of G4 structures is an emerging field of research (Seifert, 2018). *In vitro* methods have allowed for the determination of biochemical parameters like folding, stability, and protein-binding partners for G4 structures found in many biological systems (Tarsounas & Tijsterman, 2013, Bhattacharyya *et al.*, 2016, Asamitsu *et al.*, 2019). Analysis of nucleotide mutations within the G4 sequence has shown that each individual G:C base pair is necessary for *pilE* G4 structure formation and pilin antigenic variation, but thatT:A base pairs within the sequence, which are not required to form the structure, are dispensable for pilin Av (Figure 1A) (Cahoon & Seifert, 2009).

Pilin Av recombination initiation depends on a promoter located adjacent to the G4-forming sequence that initiates transcription of a *cis*-acting, noncoding RNA (sRNA) that starts within the G4 sequence (Figure 1A) (Cahoon & Seifert, 2013). Pilin Av does not occur if the sRNA is inverted or the DNA strand encoding the sRNA is switched (Cahoon & Seifert, 2009, Cahoon & Seifert, 2013). Pilin Av is also blocked when the G-rich DNA is switched to the template strand and the sRNA is transcribed from the normal strand (Cahoon & Seifert, 2013). The sRNA is transcribed on the leading strand of replication and is located approximately 280 kb away from the origin of replication (Tobiason & Seifert, 2006).

It is likely that the G-rich RNA transcript found opposite to G4 motif forms an RNA:DNA hybrid (R-loop) (Duquette *et al.*, 2004). R-loops occur when a C-rich template DNA strand forms stable base pairs with a G-rich RNA transcript (Roy & Lieber, 2009).=While R-loops are formed during essential cellular processes like plasmid replication initiation and immunoglobulin class switch recombination (Dasgupta *et al.*, 1987, Reaban & Griffin, 1990, Yu *et al.*, 2003), they can cause genomic instability if not properly resolved. The RNase H enzyme family functions to degrade the RNA in R-loops in order to maintain genomic stability (Kogoma, 1986). If R-loops are not efficiently removed from the DNA by RNase H enzymes, they cause genomic instability by impeding transcription or replication (Santos-Pereira & Aguilera, 2015, Aguilera, 2002, Tuduri *et al.*, 2009). DNA double strand breaks induced by R-loop formation are often correlated with chromosome translocations and cancers (Sollier & Cimprich, 2015, Helmrich *et al.*, 2011). Mutation of R-loop resolving

helicases, like RNase H, can result in increased R-loop formation and neurodegenerative disorders such as amyotrophic lateral sclerosis type 4 (Chen *et al.*, 2004).

The *pilE* G4-associated sRNA (renamed *gar* here) is predicted to form an R-loop that functions to keep the G-rich DNA strand free, allowing the G4 structure to form, and/or may have a more direct role in the pilin Av initiation. There is evidence that RNase H controls R-loop formation, and thereby affecting Av in the eukaryotic parasite, *Trypanosoma brucei. T. brucei* is the causative agent of African sleeping sickness and utilizes Av to alter a variable surface glycoprotein (VSG) (McCulloch *et al.*, 2015). R-loops form near VSG switch regions and upon deletion of the gene encoding RNase HI, R-loops near VSG are increased and VSG variation is increased (Briggs *et al.*, 2018, Prister & Seifert, 2018).

Detecting G4 structures within cells has been a barrier to demonstrating that these structures have distinct biological roles in cells (Seifert, 2018). We have used a previously described G4 recognizing monoclonal antibody (MAb) to specifically quantify the *pilE* G4 structure within Gc using chromatin immunoprecipitation (ChIP), and the R-loop specific S9.6 Mab in R-loop ChIP to quantify the *pilE gar*-mediated R-loop. We find that sRNA transcription initiation is a rate-limiting step for pilin Av, since reduced sRNA transcription reduces *pilE* G4 structure levels and pilin Av frequencies. While *gar* does form an R-loop, modulating R-loop stability by regulating RNase HI expression in Gc does not alter *pilE* G4 or pilin Av frequencies. We detected pilin Av frequencies using long read Pacific Biosciences amplicon sequencing and a custom analysis software (Ozer *et al.*, 2019). These results show that the process of sRNA transcription is rate-limiting for G4 structure formation and pilin Av, while R-loop stability does not alter pilin Av.

Results

Altering the frequency of G4 sRNA transcription initiation alters pilin Av frequencies and G4 structure formation

It was previously reported that transcription of *gar* is required for pilin Av, since mutation of the −10 region of the *gar* promoter ($garP_{-10}$) abrogated Av and mutation of the −35 promoter region ($garP_{-35}$) lowered Av frequencies measured with a surrogate phase variation assay (Figure 1A) (Cahoon & Seifert, 2013). While it was reported that the G4 motif is conserved among Gc and Mc strainsthat undergo pilin Av (Cahoon & Seifert, 2009), we analyzed the conservation of the sRNA promoter sequence. We found that the −35 promoter element is completely conserved among the common lab strains FA1090, MS11, MC58 and 8013. There is one or two bases different in the middle of the −10 promoter element (Figure 1B). The first two and last base of the −10 element are the most conserved based on sigma70 promoter analysis in *Escherichia coli*, and since all the promoters conform to this consensus, we believe all will be functional to promote *gar* transcription (Rangel-Chavez *et al.*, 2017). Some Mc strains (class II strains) possess a different non-variable *pilE* gene located in a different part of the genome, and the G4 motif and sRNA are not present in these strains (Cahoon & Seifert, 2009, Wormann *et al.*, 2014). Attempts to visualize the sRNA by northern blot were unsuccessful, possibly due to low abundance of the sRNA and the lack of a transcriptional terminator leading to the production of many different sRNA species (Quillin *et al.*, 2018). To determine *gar* abundance, we performed qRT-PCR with *gar* specific

primers that amplify within the first 60 nucleotides of the sRNA transcript. The *garP*$_{-10}$ mutation greatly reduced transcript levels near the background levels of the no reverse transcriptase controls. The *garP*$_{-35}$ mutation had sRNA levels of about 30% of the parental strain (Figure 1C).

The *garP*$_{-35}$ mutation was previously reported to lower pilin Av rates using the pilus dependent colony morphology change (PDCMC) assay in an inducible *recA* background (*recA6*) (Cahoon & Seifert, 2013). Pilin Av frequencies of *garP*$_{-10}$ and *garP*$_{-35}$ mutants without a regulatable *recA*(parental strain, FA1090) were determined by an novel method using PacBio sequencing (Ozer *et al.*, 2019). All starting strains were confirmed to have the same, predominate 1–81–S2 *pilE* sequence by Sanger sequencing of each progenitor colony used to generate the progeny used for PacBio sequencing, although since pilin Av was unregulated each founder colony would start with different amounts of variant *pilE* subpopulations that were below the level of detection. At least 1000 colonies were isolated after ~19–20 generations of growth from two biological replicates, and total genomic DNA was extracted for each sample (Rotman *et al.*, 2016). The *pilE* genes were amplified with pairs of primers, each with a different SMRT PacBio barcode for each strain. Individual amplicon sequences were determined by repetitive PacBio SMRTbell sequencing to reduce sequencing errors. The resulting sequences were de-multiplexed, filtered for quality, and analyzed to determine the pilin Av frequency for each strain (Ozer *et al.*, 2019). The parental strain FA1090 had an average pilin Av frequency of 18.1%, the *garP*$_{-35}$ strain had a frequency of 6.7%, and the *garP*$_{-10}$ mutant strain had a frequency of 0.1% (Table 1 and Supplemental Table 1). These pilin Av frequency values ascertained by PacBio sequencing are the first to use an unregulated Gc strain and confirmed the effect of the *gar* promoter mutations to previous results using different sequencing technologies and an IPTG-regulated strain (Cahoon & Seifert, 2013, Rotman *et al.*, 2016). These results confirm that that *gar* transcription initiation is rate-limiting for pilin Av.

An extended *gar* transcript is not required for pilin Av

The sRNA *gar* does not encode a defined transcription termination sequence and the transcript dissipates along the genome (Quillin *et al.*, 2018). We introduced a Rho-independent terminator of *trp* from *E. coli* 84 bases downstream of the transcriptional start site to specifically halt transcription elongation (Reynolds *et al.*, 1992). The efficiency of termination was determined by qRT-PCR using primers designed to amplify within the sRNA transcript and after the terminator sequence. The 84nt sRNA transcriptional terminator read through was 17% ± 6% (average ± SD), which is within the normal efficiency of Rho-independent termination (Reynolds *et al.*, 1992). Two additional strains were constructed introducing the same terminator that would produce shorter transcripts to define the transcript necessary for Av (Figure 1D). These mutants were analyzed for pilin Av frequencies using PacBio sequencing and the 72nt and 32nt *gar* transcript strains produced pilin Av frequencies of 16.9% and 17.6%, respectively. Since reduced transcription in the *garP*$_{-35}$ mutant resulted in reduced pilin Av frequencies, we conclude that the small amount of read through beyond the terminator sequence was not contributing to pilin Av. We conclude that terminating the majority of transcription 32 nucleotides after initiation is

sufficient for the *gar* transcript to promote pilin Av, and that the extended *gar* transcript plays no role in the process.

RNase HI is encoded by NGO1162 and degrades RNA from R-loops

To determine whether the *pilE* G4 sRNA forms a stable R-loop with the template strand, we used ChIP with the S9.6 Mab that recognizes RNA:DNA hybrids (Hu *et al.*, 2006, Chan *et al.*, 2014). qPCR was used to quantify the immunoprecipitated DNA of the G4 sRNA region. As a negative control we also quantified the immunoprecipitated DNA ofan irrelevant ectopic site that does not have an abundant transcript and, thus, is predicted to have none or low levels of R-loops (Mehr & Seifert, 1998). As a positive control, we also conducted R-loop ChIP of the 16S RNA, as this is a very abundant transcript (Supplemental Figure 1). A polyclonal IgG mouse antibody was used to detect nonspecific, antibody-dependent Chip signals. Mab S9.6 ChIP demonstrated that stable R-loops exist at the G4 sRNA region. Importantly, the R-loop signal was reduced is gar_{P-10} and $-garP_{-35}$ mutants (Figure 2A and Supplement Figure 1).

Since RNase H nucleases are the major means to remove R-loops, we asked whether modulating RNase H levels might influence *gar* R-loop stability. Gc encodes an RNase H1 ortholog (*rnhA)* at the FA1090 locus NGO1162 and an RNase HII ortholog (*rnhB*) at the locus NGO1789. An insertional mutant of *rnhB* showed no defects in growth, no other obvious phenotypes, and had parental levels of pilin Av when measured by the surrogate PDCMC assay (Supplemental Figure 2).

Multiple attempts to disrupt the *rnhA* gene were unsuccessful, even in a $garP_{-10}$ mutant strain, showing that the lethality was not due to the presence of the *gar* sRNA. To overcome the potential lethality of the *rnhA* mutation, an IPTG-inducible copy of the *rnhA* gene driven by the *lac* promoter, was inserted into an intergenic region elsewhere on the Gc chromosome (*nics)* (Mehr & Seifert, 1998). The mutated *rnhA* gene was introduced into this strain at the NGO1162 locus with IPTG in the growth medium, to create the strain *rnhA*+$nicsP_{lac}$::*rnhA*. The *rnhA*+$nicsP_{lac}$::*rnhA* strain grew without IPTG in the growth medium, albeit more slowly than the parent strain. We reasoned that the ability to grow without *rnhA* induction was due to residual transcription from the repressed *lac* promoter. qPCR analysis confirmed that the *rnhA*+$nicsP_{lac}$::*rnhA* strain produced ~30% of the *rnhA* transcript of the parental strain (Figure 2B). The *rnhA*+$nicsP_{lac}$::*rnhA* strain showed a dose-dependent response to IPTG in the growth medium with 0.005 mM IPTG providing *rnhA* transcript levels equivalent to the parental FA1090 strain and up to a 50-fold overexpression of *rnhA* transcriptswith 1 mM IPTG (Figure 2B).

To determine whether *rnhA* levels affect R-loop stability, Gc was grown with and without IPTG to allow low and high levels of RNase HI expression. Mab S9.6 ChIP showed that when the *rnhA*+$nicsP_{lac}$::*rnhA* strain was grown without IPTG, R-loop levels were 10-fold higher than parental levels (Figure 2). R-loop levels reached parental levels when *rnhA* +$nicsP_{lac}$::*rnhA* was grown with 1 mM IPTG, which causes overexpression of *rnhA* (Figure 2C). The negative controls confirmed that the S9.6 ChIP was specific since S9.6 did not pull down the ectopic locus (Supplemental Figure 3A). Additionally, the R-loop signal was lost after treating ChIP samples with *E. coli* RNase HI *in vitro* after pulldown, showing that S9.6

was detecting R-loops and not non-RNA structures (Supplemental Figure 3B) (El Hage *et al.*, 2014). *rnhB* modulation did not alter R-loop levels and was therefore not analyzed further (Supplemental figure 3C). These results show that RNase HI does process the *pilE* G4 R-loop, but that RNase HI overexpression did not reduce *pilE* G4 R-loop levels below that of the parental strain.

ChIP can detect and quantify the genomic *pilE* G4

It has been very difficult to detect or quantify G4 structures in cells. Many of the techniques used are limited in their inability to differentiate between G4 structures present within the cell and G4 structures that form after cell lysis and DNA extraction, particularly because the DNA is often denatured during extraction. To avoid this issue, we developed a G4 ChIP assay to detect and quantify G4 levels within the bacterial cell. After testing several MAbs and recombinant MAbs reactive to the G4 structure, we determined that the MAb, 1H6, which detects multiple G4 structures was the most sensitive antibody in our bacterial system (Henderson *et al.*, 2014). To ensure that the 1H6-ChIP was detecting the presence of the *pilE* G4 within the bacteria, a G4 mutant in which 3 guanine tracts were disrupted, but the total number of G residues was retained, was used as a negative control. Additionally, a positive G4 ChIP control was performed using a mutant strain inactivated for two helicases, RecQ and Rep, active in unwinding G4s *in vitro* (Cahoon *et al.*, 2013). The *recQ rep* double mutant had smaller colonies (Cahoon & Seifert, 2009). We detected a loss of the G4 ChIP signal in the G4 mutant and an increase in the *recQ/rep* mutant (Figure 3) demonstrating that this G4 ChIP assay is specific for the *pilE* G4 and detects and quantifies G4s existing within the bacterial cell.

The *gar* ranscript is required for G4 formation and stability, but R-loop stability does not influence the *pilE* G4

The discovery of the requirement for transcription of the *gar* sRNA led to several hypotheses. First, transcription is necessary to open the DNA duplex allowing for the single stranded G-rich strand to fold into a G4 structure (Cahoon & Seifert, 2009). A second hypothesis, not mutually exclusive to the first hypothesis, is that the R-loop itself plays an active role in the process of pilin Av. To test the first hypothesis, we used the *gar* −10 and −35 promoter mutants. As expected, the $garP_{-10}$ mutation that disrupts sRNA transcription and pilin Av also prevents the G4 ChIP signal (Figure 4A). The $garP_{-35}$mutation, which produces an intermediate level of sRNA transcript (Figure 1B), reduced G4 levels (Figure 4A). Taken together these results confirm that transcriptional melting og the DNA duplex is the primary reason that pilin Av relies on the sRNA (Figure 4A and Supplemental Figure 4A).

Since down regulation of *rnhA* expression leads to an increase in R-loop levels, we asked whether the increased *gar* R-loop stability results in increased G4 formation. In the mutant strain where R-loop levels are high (*rnhA*+$nicsP_{lac}$::*rnhA* in the absence of IPTG), G4 levels were similar to the FA1090 parent (Figure 4B and Supplemental Figure 4B). When *rnhA* +$nicsP_{lac}$::*rnhA* was grown in the presence of 1mM IPTG, *rnhA* was over expressed, but R-loops levels were similar to parental levels, and G4 levels wereslightly reduced to 0.67 fold

of parental levels. This result suggests that while transcription of the *gar* sRNA is necessary for G4 formation, that increased stability of the R-loop is not important.

R-loop stability does not influence pilin Av frequencies

While increased R-loop stability did not change G4 levels (Figure 4), it was possible that changes in R-loop levels or R-loop stability might still alter pilin Av frequencies. A PacBio sequencing assay was used to determine the pilin Av frequency of various strains (Ozer *et al.*, 2019). Deep sequencing is the best method for determining Av frequency when the strains tested have different growth rates, a factor relevant to this work, as the *rnhA* +$nicsP_{lac}$::*rnhA* strain exhibits slower growth in the absence of IPTG. Additionally, the longer reads generated during PacBio sequencing allows for the *pilS* silent copy donors to be quantified and defines the types of recombinants produced (Supplemental Figure 5 and 6) (Ozer *et al.*, 2019).

Pilin Av frequencies were compared between the *rnhA*+$nicsP_{lac}$::*rnhA* strain with varying RNase HI levels produced by growth on different amounts of IPTG (0.0, 0.005 or 1 mM IPTG). Pilin Av was abrogated when the G4 motif was mutated as reported previously (Cahoon & Seifert, 2009). In mutant strains where the sRNA promoter is altered, $garP_{-10}$ and $garP_{-35}$, pilin Av frequencies were similarly reduced, confirming result from the PDCMC assay (Cahoon & Seifert, 2013). However, pilin Av frequencies were not substantially affected by modulating the R-loop levels through the differential expression of RNase HI (Table 1 and Supplemental Tables 1 and 2). These results allow us to conclude that increasing R-loop stability does not affect pilin Av frequencies.

Discussion

We have determined that transcription of the G4 sRNA is the primary driver of pilin Av initiation. Mutation of the G4 sRNA promoter elements had previously been shown to lower or completely abrogate pilin Av, measured by the surrogate PDCMC assay (Cahoon & Seifert, 2013). In addition, a previously published 454 sequencing assay confirmed that a mutation in the −35 region of the sRNA promoter reduced pilin Av frequencies (Rotman *et al.*, 2016). We have confirmed and extended these results to show that sRNA transcription initiation is rate limiting for pilin Av. We used a novel PacBio amplicon sequencing method to test for pilin Av frequencies in our different strains. A similar methodology could be used with Nanopore MinION sequencing which has lower rates of insertions and deletions, 4.9% and 7.8% respectively (Jain *et al.*, 2015). However, PacBio CCS allows for multiple sequencing passes of the same DNA molecule, reducing the error rate over multiple reads. Moreover, while the sRNA transcript does not end at a specific terminator sequence, and transcript abundance appears to dissipate as downstream distance from the promoter sequence increases, only 32 nucleotides of the sRNA transcript is required for normal levels of pilin Av. The full activity of this shortened transcript in pilin Av suggests that the *gar* sRNA acts mainly opposite to the G4 motif and there is no function for extended sRNA transcription, at least for pilin Av. Since there is no gene downstream of the G4 sequence, there may not have been a reason to terminate this low abundance transcript. We have been unable to delete the A-rich sequence adjacent to the G4 forming motif (Figure 1) for

unknown reasons, so we cannot rule out a role for this sequence within the sRNA in pilin Av. Since the G4 structure cannot form when DNA is double stranded, the DNA duplex needs to be opened by a helicase (Hardin *et al.*, 1992, Li *et al.*, 2002). The breaking of hydrogen bonds could be catalyzed by a dedicated helicase, or any of the helicase activities required for DNA replication, transcription, repair or recombination. Based on the phenotype of the −10 promoter mutant, we conclude that it is transcription by the house-keeping polymerase that opens the duplex to allow for G4 formation.

In all types of cells, R-loops are often found opposite G4 forming sequences, since G-rich RNA preferentially binds to C-rich DNA (Gyi *et al.*, 1996). We confirmed an R-loop was forming in the G4 sRNA region by R-loop ChIP and confirmed that R-loop formation depends on G4 sRNA, *gar*, transcription. Understandably, in the −10 mutant defective for transcription initiation, there is no R-loop formation. Similarly, when transcription efficiency is low in the −35 mutant, R-loop levels are reduced. To understand how changes in R-loop levels or R-loop stability may influence pilin Av, we created the *rnhA*+*nicsP*$_{lac}$*::rnhA* strain, allowing for modulation of *rnhA* expression with IPTG. However, in strain *rnhA* +*nicsP*$_{lac}$*::rnhA* in the absence of IPTG, wherein R-loop levels were increased 10-fold above parental levels, there was no increase in G4 levels, showing that increased R-loop abundance does not influence G4 formation or stability. Most importantly, in this same strain under the same conditions of increased R-loop stability, Av frequencies were unaffected. Without IPTG, this strain grows slowly, and we presume that there is an increase in R-loops throughout the genome. An increase in the stability of all R-loops may be create genomic instability influence the pilin Av process by recruiting essential recombination and repair factors. Surprisingly, when *rnhA* was overexpressed, R-loop levels were not significantly different from the parental strain. This result shows that, even though lowering RNase HI increases sRNA R-loop abundance, there exist R-loops that remain unaffected by parental and overexpressed levels of RNase HI. The most likely explanation for this effect is that RNase HI is not acting on its own to control R-loop stability, and that activity on the sRNA is linked to other processes such as DNA replication. It is also possible that a small portion of the sRNA can form a structure that is resistant to digestion, such as triplex DNA or dsRNA (Hartono *et al.*, 2018). Interestingly, when *rnhA* was overexpressed, there was a slight decrease in G4 levels, even though overall R-loop levels remained unchanged. This effect may be due to an independent effect on the bacterial cell of expressing the RNase HI 50-fold above normal expression levels. As this small decrease in G4 levels during *rnhA* overexpression does not affect pilin Av frequencies, this discrepancy may reflect the fact that ChIP is not a perfectly quantitative assay.

Gc also encodes the RNase HII family enzyme *rnhB* (NGO1789). A *rnhB* mutant and complement had similar growth phenotypes and no effect on pilin Av as measured by PDCMC. While RNase HII family enzymes can act on R-loops, they are more often involved in removing ribonucleotides during replication (Itaya, 1990, McDonald *et al.*, 2012). The lack of an *rnhB* phenotype suggests that misincorporated ribonucleotides are not involved in pilin Av. Since low *rnhA* expression leads to a large increase in R-loop levels, we conclude that in Gc, RNase HI is the primary enzyme functioning to remove R-loops.

Though critical for pilin Av and other microbial cellular processes (Seifert, 2018), G4 levels within a bacterial cell have not been previously reported. Therefore, we developed a G4 ChIP assay to understand how the G4 functions during pilin Av. Using G4 ChIP, and G4 and helicase mutants as negative and positive controls, respectively, we were able to quantify a cellular G4-specific signal. Since the 1H6 Mab also has affinity to T-rich (thiamine-rich) DNA in addition to G4 structures (Kazemier *et al.*, 2017), and there is a T-rich sequence near the G4 motif (Figure 1), it was possible that we were precipitating this T-rich sequence and not the G4 structure. However, the T-rich region is present in both the G4 mutant and parent strain, yet the G4 signal is lost in the G4 mutant (Figure 3). The 1H6 MAb also has affinity to Ts contained within the loops of a G4 structure, which are present in the *pilE* G4 (Kazemier *et al.*, 2017). Whether the 1H6 MAb detects the G4 structure itself or the specific structure of the G4 loop Ts is immaterial since the MAb only pulls down G4 DNA and not G4 mutant DNA. Additionally, G4 ChIP signals increased when two G4 interacting helicases (RecQ and Rep) were inactivated. If G4 folding was only occurring during the nucleic acid extraction or the ChIP protocol, we would expect the G4 signal to be similar between the double helicase mutant and the parent strain. Therefore, G4 ChIP with MAb 1H6 reliably detects and quantifies G4s within the cell.

The critical role for the *gar* sRNA in Av appears to be opening duplex DNA through transcription, although there are a few other plausible roles for the sRNA in pilin Av. In all of the strains that allow sRNA transcription there is some level of R-loop formation and the R-loop would still prevent the C-rich strand from reannealing to the G-rich strand. Thus, R-loop formation and limited stability may still be important for G4 folding. The sRNA could have an additional role in pilin Av by directly engaging with the G4 DNA structure. The *gar* transcript contains three G-tracts and one or more of the tracts could participate in an intermolecular G4 structure creating a DNA/RNA G4 (Xu *et al.*, 2012). We consider this hypothesis unlikely since individual mutation of 11 of the 12 of the G4 G:C base pairs abrogates Av, and mutation of the 12^{th} lowers pilin Av since a 13^{th} G can compensate for its loss (Cahoon & Seifert, 2009). If the sRNA participated in an alternative G4 structure, all four G-tracts may not be required for pilin Av, particularly since the first tract is not present in the sRNA. The sRNA or R-loop formation could also be recruiting factors to *pilE* to enhance recombination, but this idea remains to be tested. The exact sequence requirement of the G4 transcript has not been determined. The G4 motif cannot be mutated without altering the G4 DNA structure. The A-rich region of the sRNA could also play an additional role in pilin Av. It should be noted that since transcription is required for R-loop formation, we have not directly tested for a role of the R-loop in pilin Av without transcription opening the duplex and allowing formation of the G4 structure.

We conclude that sRNA transcription is rate limiting for G4 formation and although R-loops do form at this location, increased R-loop stability is dispensable for pilin Av. We hypothesize pilin Av is initiated through a DNA break caused by stalled chromosomalreplication (Figure 5). Either the G4 or R-loop upstream of *pilE* could participate in creating a DNA break. However based on these results; it is more likely that the G4 blocks replication, because G4 levels and Av occur similarly when R-loop levels are high or low. If R-loops were participating in genomic instability, we would predict that increased R-loops in the Rnase HI under expressing strain would cause an increase in Av

frequency through additional DNA breaks. However, the pilin Av frequency when R-loop levels are increased is slightly lower compared to normal levels of R-loops. R-loops have been implicated in many pathologies including oncogenesis by way of chromosomal translocations and neurological syndromes (Bhatia *et al.*, 2014, Groh *et al.*, 2014, Haeusler *et al.*, 2014). Since most stable R-loops are G-rich, the role of G4s and other alternative DNA and DNA/RNA structures has not always been investigated. The identification of roles for R-loops in mediating positive biological processes is less well established than detrimental roles. R-loops are necessary for the initiation of replication in some plasmids and mitochondrial DNA (Xu & Clayton, 1996, Fukuoh *et al.*, 1997). R-loops are also important for *Trypanosoma brucei* VSG Av since the loss of RNase HI influenced Av frequencies (Briggs *et al.*, 2018). R-loops are also implicated in Ig class switch, where the RNA G4 aids recruitment of the cytidine deaminase, which leads to recombination (Zhang *et al.*, 2014, Zheng *et al.*, 2015). We predict that other diversity generation systems, many that have G-rich segments associated with them (Walia & Chaconas, 2013, Giacani *et al.*, 2012, Glover *et al.*, 2013, Smargiasso *et al.*, 2009, Briggs *et al.*, 2018, Beaume *et al.*, 2013), may also depend on R-loops and associated G4s to induce programmed recombination.

Methods

Gc Strains and Growth Conditions

Bacterial strains used in this study were derivatives of the FA1090 clinical isolate 1–81–S2 that was re-isolated from a symptomatic, human-volunteer infection (Seifert *et al.*, 1994). All gonococcal strains carried the 1–81–S2 pilus variant sequence as determined by Sanger sequencing (Seifert *et al.*, 1994). The *Escherichia coli* strain DH5α was used for expression of mutant constructs in plasmid cloning vectors and grown on Luria-Bertani Broth. *N. gonorrhoeae* strains were maintained on Gc medium base (Difco) plus Kellogg supplement I (22.2 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase) and Kellogg supplement II (1.23 mM $Fe(NO_3)_3$) (Kellogg *et al.*, 1968). When noted, strains contain the modified *recA* gene, *recA6*, which places the endogenous gene under the control of the *lac* promoter, allowing for IPTG-dependent regulation of pilin Av (Seifert, 1997). When stated, Gc were propagated in liquid culture using a previously described method (Stohl *et al.*, 2005) to obtain a uniform population in mid-exponential growth phase.

Mutant Strain Construction

KOD polymerase (Novagen) was used to amplify the upstream and downstream regions of *rnhA*, encoding Rnase HI, (FA1090 locus, NGO1162, Genbank ID) with phosphorylated primers rnhA-usF and rnhAusR-NotI, and rnhA-dsR and rnhAdsF-NotI (Supplemental Table 3 and 4). The 3′ primer of the upstream region (rnhAusR-NotI) and 5′ primer of the downstream region (rnhAdsF-NotI) contained complementary sequences to introduce a NotI restriction site. Splicing overlap extension PCR (SOE-PCR) was used to combine the upstream and downstream regions, creating a mutant construct containing a NotI restriction site where the *rnhA* coding sequence had been. *rnhB* (FA1090 locus, NGO1789, Genbank ID) was deleted and complemented similarly with primers r2usF and r2usRkpnI, and r2dsR and r2dsFkpnI deleting *rnhB* and inserting a KpnI site. These constructs were cloned into pSMART LCAmp (Lucigen 40300–2) following the manufacturer's instructions and

introduced into *E. coli* DH5α by electroporation. The *rnhA* and *rnhB* mutant constructs-containing pSMART LCAmp sequence, pSMART-Δ*rnhA* and pSMART-Δ*rnhB*, were confirmed by Sanger sequencing. Both plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen 27104) according to the manufacturer's instructions, digested with NotI or KpnI (NEB R0189S), and gel-purified using the QIAquick gel extraction kit according to the manufacturer's instructions (Qiagen 28115). The chloramphenicol resistance (CmR) gene was digested from plasmid pHSS6-Cm2–9 using NotI (Seifert *et al.*, 1990), gel purified using the QIAquick gel extraction kit, introduced into the NotI site of pSMART-Δ*rnhA*, and ligated using T4 DNA Ligase (ThermoFisher EL0014). The KanR gene from pBSL86 (Alexeyev, 1995) was digested with KpnI and cloned into pSMART-Δ*rnhB*. The pSMART-Δ*rnhA*-CmR and pSMART-Δ*rnhB*-KanR ligation products were electroporated into *E. coli* DH5α selecting for chloramphenicol resistance at (50 ug/ml) and Kanamycin resistance at (50 ug/ml) respectively.

The RNase HI complementation construct was made using primers rnhAFPacI and rnhARPmeI, containing PmeI and PacI cut sites, to amplify the *rnhA* gene in strain FA1090. The amplified PCR product and recipient plasmid pGCC4 (Mehr & Seifert, 1998) were both cut with PmeI and PacI, then ligated together with T4 DNA ligase. The pGCC4-*rnhA* complementation plasmid was isolated using the QIAprep Spin Miniprep Kit and the *rnhA* insertion sequence was confirmed by Sanger sequencing using primers Lctpout and LacPFor. *rnhB* was complemented similarly with primers R2FPacI and R2RFseI and inserted into pGCC4. Plasmid constructs pSMART-Δ*rnhA*-CmR and pGCC4-*rnhA*, and pSMART-Δ*rnhB*-KanR and pGCC4-*rnhB* were introduced into the Gc chromosome by natural transformation as described previously (Stein 1991) and confirmed by PCR and Sanger sequencing.

Rho-independent terminator mutants of the G4 sRNA, G4 mutation, and $garP_{-10}$ were synthesized as gBlocks (IDT) (Reynolds *et al.*, 1992) . All terminator mutant gBlocks were cut with PacI and inserted into the PacI and EcoRV cut site of the *pilE* plasmid containing the region between USS2-pilArev2 (Cahoon & Seifert, 2009) and transformed into DH5α. The transformants were confirmed by Sanger sequencing and then transformed into the chromosome of FA1090 *recA6* and FA1090. Kanamycin resistant (KanR) colonies were selected and transformants were verified by PCR and Sanger sequencing with KanFor and RT-G43R. The G4 mutant was cloned similarly in the *pilE* plasmid containing the region between USS2-pilArev2 and was transformed into FA1090.

Mutation of the −10 box of the G4 sRNA promoter was synthesized as a gBlock (IDT). The gBlock was cloned into vector pBlunt-TOPOII (Thermo Fisher). The plasmid was then extracted and transformed into the parental FA1090, the *rnhA* mutant, and *rnhB* mutant strains. Transformants that no longer produced nonpiliated blebs over extended growth on solid medium (Seifert, 1997) were screened by PCR and subsequent sequencing to identify G4 sRNA −10 mutants.

Mutation of the −35 promoter element in FA1090 was performed by isolating genomic DNA from the *recA6* −35mutant (Cahoon & Seifert, 2013). The genomic DNA was transformed into FA1090 by spot transformation, and Kan resistant colonies isolated and sequenced.

qRT-PCR to detect sRNA and mRNA Expression

For transcriptional terminator experiments, strains were grown in liquid medium to mid log phase (OD_{A550}~0.5), back-diluted to an OD_{A550} of 0.07 and grown for four hours. Since low expression of RNase HI causes a growth defect, RNA was collected from all strains after overnight growth on solid media instead of liquid growth. For all Gc strains for which *rnhA* transcript expression would be tested by quantitative reverse transcription PCR (qRT-PCR), all strains were grown on GC agar medium overnight and lawns were swabbed for qRT-PCR. After growth on either liquid or solid GCB medium + Kellogg's supplements I and II, bacteria were treated with RNA-Protect reagent (Qiagen 76526) and pelleted by centrifugation. Total RNA was purified using the RNeasy kit (Qiagen 74104) according to the manufacturer's instructions. Purified RNA was treated with RQ1 DNase (Promega M6101) to remove contaminating genomic DNA, then further purified with the RNeasy kit. RNA was reverse transcribed using Superscript III polymerase (Invitrogen 18080093). To assure there was no genomic DNA contamination in the samples undergoing RT-qPCR, no reverse transcriptase controls were included with all samples tested. cDNA was amplified using the iQ SYBR Green Supermix (BioRad 170–8882) according to the manufacturer's instructions with the following cycling parameters: one cycle, 95°C 3 min.; 40 cycles, 95°C 10 s, 58°C 30 s (Anderson & Seifert, 2013). Relative transcript abundance was determined using the ΔΔCt method of Schmittgen and Livak (Schmittgen & Livak, 2008). Housekeeping gene *omp3* was used as the reference gene internal control (Stohl *et al.*, 2005). All amplifications yielded a single product based on melting temperature analysis, all primer sets were optimized to assure uniform primer efficiency (Schmittgen & Livak, 2008). Expression results for mutant strains are expressed as the mean fold change in transcript abundance ± one standard deviation compared to the FA1090 *recA6* or FA1090 parental strain (Anderson & Seifert, 2013).

The efficiency of the *E. coli* Rho-independent terminator sequence in halting sRNA transcription was measured by qRT-PCR using internal primers (RT-G4start and RT-midfor) within the first part of the sRNA and external primers designed to detect the sRNA sequence after the terminator sequence (RT-G4start and RT-termfor). The ratio of read-through sRNA was compared to total transcript levels by comparing the external primer set to the internal primer set. (RT-G4start and RT-midfor, RT-G4start an RT-termFor). *rnhA* transcript levels were determined using primers RT-rnhAF2 and RT-rnhAR2.

PDCMC Assay

The surrogate Av assay was performed as previously described (Sechman *et al.*, 2005), with exception that the strains did not contain a regulatable *recA* gene. Therefore, the *pilE* sequence of each seeding colony was determined by sanger sequencing to ensure each assay started with the same *pilE* sequence (Seifert *et al.*, 1994). A single colony (confirmed to be 1–81–S2)was spread on GCB agar plates and colony morphology tracked starting at 22 hours. Ten fully piliated colonies were selected and followed for 6 hours with scoring every two hours. Each bleb accounted for one point and four or more blebs was scored as a four.

RNA:DNA Hybrid ChIP Assay

ChIP was performed using confluent lawns of Gc grown overnight at 37°C with 5% CO_2 on GCB agar + Kellogg supplements I and II with or without 1 mM IPTG. Total nucleic acids were extracted using phenolchloroform without exposure to heat (Wilson, 2001). 100 ug of DNA was then digested with 1 ul Mung Bean Nuclease (Promega M4311) for 30 min at 37 degrees C to stabilize R-loops (Wahba *et al.*, 2016, Mehta & Laimins, 2018). DNA/RNA was ethanol precipitated in 50 ul 7.5M ammonium acetate and 250 ul ethanol at −80°C for 1 hour and centrifuged for 15 min at 15000xg at 4°C. The pellet was washed in 70% ethanol and the desiccated pellet was dissolved in 100 ul ddH2O. DNA was sonicated with a Diagnode Bioruptor with the settings: 30 seconds on and 30 seconds off, for 30 min at 4°C.

20 ul per sample of Protein-A Dynabeads (Invitrogen) were incubated with Herring testes DNA and BSA and incubated overnight at 4C. Beads were incubated with 20 μg/ml of anti-DNA/RNA hybrid S9.6 MAb (generously provided by Dr. Nicholas Proudfoot, Oxford University) or a normal mouse polyclonal IgG control antibody (sc2025 Santa Cruz Biotechnology) at 4°C for 8 hours. Supernatant was removed and 1 ml IP buffer (1% Triton X-100, 0.1% sodium deoxycholate, 10 mM EDTA, 50 mM Tris-HCL pH 8.0) and 100 ul sheared DNA were added to each tube and mixed overnight at 4°C. Bead complexes were washed eight times with 1 mL of RIPA buffer (50 mM Hepes pH 8.0, 1 mM EDTA pH 8.0, 1% NP40, 0.7% sodium Deoxycholate, 0.5 M LiCl, and Protease inhibitor). Complexes were suspended in 1 mL of 1X TE buffer and centrifuged at 3000 rpm for 3 minutes. The complexes were treated with 50μl of elution buffer (10 mM Tris pH 8.0, 1 mM EDTA, 10% SDS) at 65°C for 10 minutes, vortexing periodically during incubation. The eluted complexes were spun for 1 minute at 15000 rpm and then transferred to fresh tubes. This process was repeated with an additional 50 ul elution buffer. The precipitated DNA was purified by using a Qiagen DNA purification kit following the manufacturer's protocol. (Mehta & Laimins, 2018, Wong *et al.*, 2010). Primers RT-G4start and RT-midFor were used to amplify the G4 region, USaspcF1 and USaspcR1 were used to amplify the ectopic site, and 16sF1 and 16sF2 were used to amplify the 16s region. The ectopic site is used as a negative control, since transcription at this site is low, the pull down of this region of the genome should be low and represent non-specific binding of the S9.6 antibody (Mehr & Seifert, 1998). 16S RNA-specific primers were used in as a positive control, as transcription in this region is consistently high enough that R-loops should always be present. All primers were determined to have similar levels of efficiency based on the procedure of Livak et al. (Schmittgen & Livak, 2008). On bead treatment with *E. coli* Rnase H (NEB) was performed twice (El Hage *et al.*, 2014).

G4 ChIP Assay

When multiple G4 specific antibodies (including BG4 (Biffi *et al.*, 2013) and an unpublished phage display G4 MAbs) were tested for G4 ChIP signal (Hansel-Hertsch *et al.*, 2018) and low background binding to DNA, only the 1H6 MAb produced a G4-specific signal. G4 ChIP was performed similarly to the previously described R-loop ChIP protocol, with the exception of not conducting the mung bean nuclease treatment. After extraction, 100 ug of DNA was digested with 10 ul BfaI (NEB). The extracted DNA was ethanol precipitated as described above. The digested DNA was added to beads blocked with either 2ug of MAb

IH6 (MABE1126 Millipore) (Henderson *et al.*, 2014) or a control mouse IgG (Santa Crux Technologies) in IP buffer, and incubated room overnight at 4C. Beads were washed eight times in RIPA buffer and complexes were suspended in 1 mL of 1X TE buffer and spun down at 3000 rpm for 3 minutes. The complexes were treated with 50 µl of elution buffer twice at 65 C. The precipitated DNA was quantified by qPCR with the primers RT-G43F and RT-G4R. The primer set used to quantify R-loop levels could be used in G4 ChIP as well, however, PCR efficiency across a G4 structure can affect the quantification. Since, we wanted to compare the parent and G4 mutant, we designed a primer set outside the G4 motif to avoid this complication.

PacBio Sequencing to determine Pilin Av Frequency

Strains were revived from frozen glycerol stocks at −80 C by streaking for overnight growth for 18 hours on GCB agar + Kellogg supplements I and II. A single colony was collected using a sterile 6 mm filter disk and dispersed in 500 ul GCBL with vigorous vortex mixing and dilutions were plated on GCB plates to obtain 200–400 colonies per plate. The remainder of the cell suspension was pelleted, washed with 1X PBS, and the bacteria were lysed in cell lysis buffer. The lysed bacterial suspension was used as a template for PCR and subsequent sequencing with primers PilRBS and Sp3A. This step ensured that all strains sequenced began with the same variant *pilE* sequence (1–81–S2) at the beginning of the experiment (Seifert *et al.*, 1994).

rnhA+*nicsP*$_{lac}$::*rnhA* without IPTG grows slower than the parent strain. To match the number of generations of the mutant strain to the parent strain during growth preceding extraction for PacBio sequencing, the strains were grown for different amounts of time that would amount to the same colony forming units (CFU) per single colony ratio (CFU/colony) for both strains, normalizing the growth state of all strains sequenced (Rotman *et al.*, 2016). The *rnhA* mutant grown for 26 hours matched the CFU/colony value of the parental FA1090 strain at 22 hours, so these respective time points were used for these strains. At least 1000 total colonies of each strain were pooled in liquid GCB medium + Kellogg Supplements I and II, from which genomic DNA was isolated using the QiaAmp kit (Qiagen 51304). The *pilE* gene was amplified using the following reaction: 1 ng genomic DNA, 20 uM dNTPs, 1X Phusion reaction buffer, 0.5 uM Primer 1, 0.5 uM Primer 2, 3% DMSO, 1 unit Phusion Hot Start Flex (NEB) Polymerase and water. The reaction was run under the following conditions: 98°C for 30 s for initial denaturation and Polymerase activation, 98°C for 10 s, 65°C for 30 s then 0.3°C reduced in each cycle, 72°C 1min, repeat cycles 30 times, final extension for 5 min at 72°C.

A unique 16 base barcode identifier for each sample was encoded by including the PacBio barcode sequence (Verhey *et al.*, 2018) on both the Forward and Reverse primers that amplified the *pilE* locus amplicon sequenced in this study (Supplemental Table 5). Amplification with the primers PilRBS-TTTCCCCTTTCAATTAGGAG and OpaERev-GGGTTCCGGGCGGTGTTTC yielded 788 bp of *pilE* sequence within the 820 bp total amplicon containing the unique barcodes. PCR products were gel extracted without Ethidium bromide exposure. Gel extraction was performed with the QiaQuick gel extraction kit (Qiagen) according to the manufacturer’s instructions, except the gel slices were

dissolved at room temperature to maintain DNA integrity. The DNA was eluted with TE buffer and pooled to obtain 300 ng of DNA per sample. Samples were submitted to the University of Maryland Genomic Resource Center, where the amplicons were cleaned with SPRI clean up, quantified and pooled into two runs. The pools underwent SMRTbell library prep and subsequent sequencing on the Pacific Biology Sequel machine SMRT cell with v3 reagents (Verhey *et al.*, 2018). Frequencies were analyzed using a custom program (https://github.com/egonozer/switchAmp) (Ozer *et al.*, 2019). Reads can be accessed under the SRA project SRP214219 with the bioproject accession number PRJNA553228 (Supplemental Table 2).

The silent copy choice for each variation event was also analyzed using the SwitchAmp program. There were four common silent copy choices among all the strains tested. The tail sequence of silent copies 2c1 and 6c1 (2c1:6c1 HV_T) was one of the common events, similar to previously published results (Criss *et al.*, 2005, Rotman *et al.*, 2016). We also observed a mosaic sequence containing both 1–81–S2 and the 2c1:6c1 tail sequence that was not observed in the negative control strains. Additionally, the use of silent copy 3c1 was common among all the strains. The forth major category of variants was multivariant, in which the read contained silent copy sequence shared among multiple copies, so a single donor could not be identified (Supplemental Figure 5 and 6) (Ozer *et al.*, 2019).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Author Summary

Neisseria gonorrhoeae, the causative agent of gonorrhea, changes one of its surface exposed structures to avoid immune detection. This process is known as antigenic variation and involves a complex process of DNA recombination. There are two alternate DNA and RNA structures that may be involved in DNA recombination adjacent to the gene that undergoes antigenic variation, a guanine quadruplex and an RNA:DNA hybrid made from a small RNA. Both of these structures can cause nicks or breaks in DNA, and this work investigates if the RNA:DNA hybrid influences the guanine quadruplex or antigenic variation. We found that reducing transcription lowers both guanine quadruplex and antigenic variation, but increasing the RNA:DNA hybrids does not change either one.

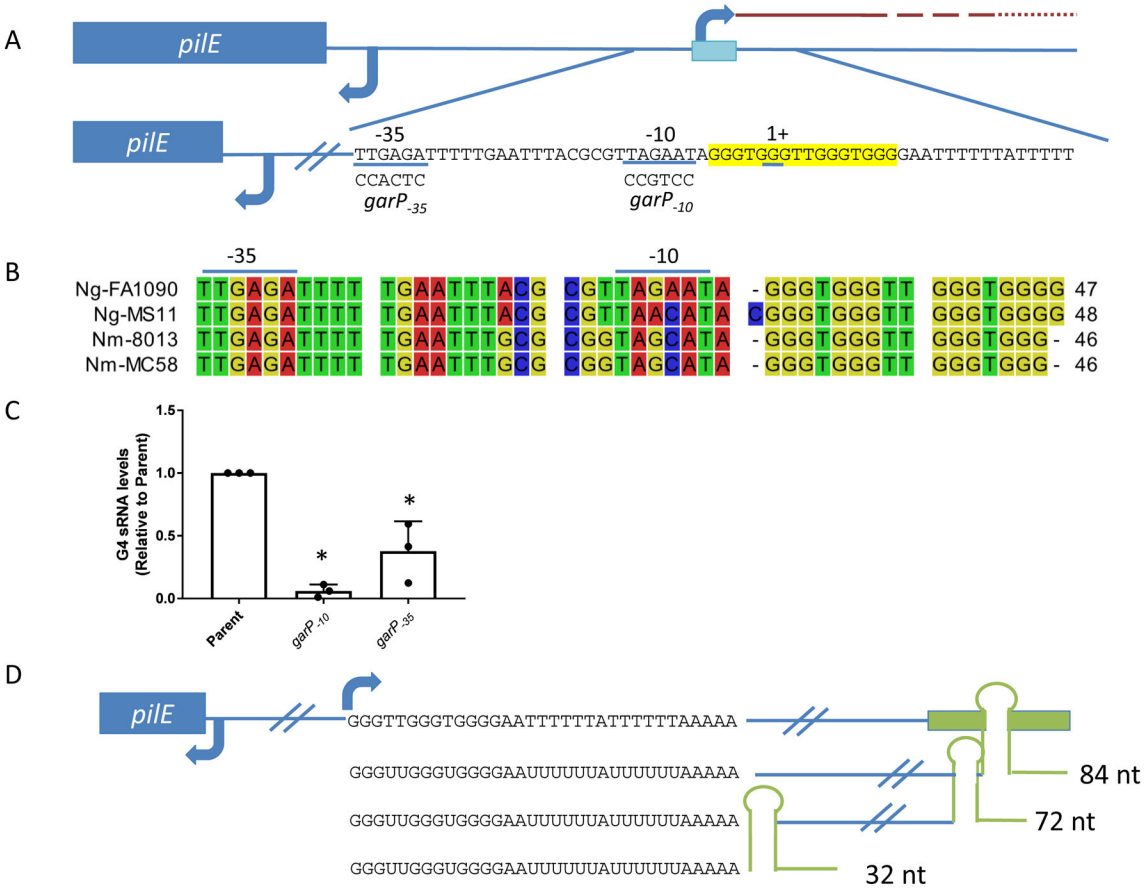

Figure 1.

gar transcript levels influence pilin Av frequencies

A. Diagram of the G4 *gar*, *pilE* gene and G4 motif (blue box and highlighted in yellow). *gar*, the *pilE* G4-associated sRNA, is long and poorly expressed, as represented by the red dashed line indicating decreasing RNA transcription. In the mutants, the $garP_{-10}$ sequence has been mutated to CCGTCC and the $garP_{-35}$ sequence has been mutated to CCACTC. The G4 sRNA initiates within the G4 motif, using the C-rich side of the DNA as the template.

B. Alignment of the −35 promoter element to the G4 motif of *common N. gonorrhoeae* (Ng) and *N. meningitidis* (Nm) strains using CLC sequence viewer. The −35 and −10 promoter elements are denoted with a line along the top.

C. Transcript levels in the parent strain (*recA6*) and mutant strains $garP_{-10}$ and $garP_{-35}$ were measured using qRT-PCR. Strains were grown in liquid medium to obtain similar growth phase to maintain RNA quality. Primers were designed to only amplify within the sRNA region. The reported value represents the mean of three biological replicates +/− one standard deviation as indicated by the error bars. There is no significant difference between the transcript levels of $garP_{-10}$ and $garP_{-35}$ but the average $garP_{-35}$ transcript levels (0.38) are 6x higher than the $garP_{-10}$ (0.06). Unpaired student t-test **p<0.01

D. Cartoon showing the *gar* DNA sequence with a terminator sequence added (Green box) and the resulting RNA products. We constructed strains to create a84nt, 72 nucleotides and 32 nucleotides terminated *gar* . The 84 nucleotides sequence was used to determine the *E.*

coli terminator efficiency. Pilin Av frequencies of the 72 and 32 nucleotides strains were determined by PacBio sequencing.

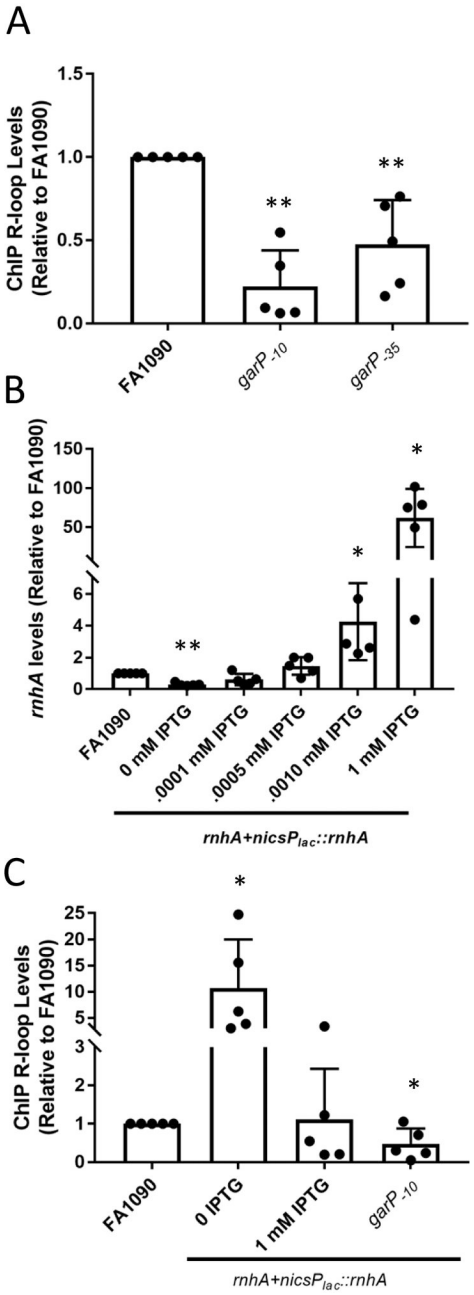

Figure 2.

Transcription and modulation of *rnhA* modulates R-loop levels

A. R-loop levels of $garP_{-10}$ and $garP_{-35}$ strains relative to the parental strain (FA1090). Relative R-loop levels are calculated by determining the percent input after ChIP pulldown for each sample and the dividing by the percent input of FA1090. The reported value represents the mean of five biological replicates (individual dots) +/− one standard deviation as indicated by the error bars. There is no significant difference between $garP_{-10}$ and $garP_{-35}$. Unpaired student t-test **p<0.01 *p<0.05

B. The *rnhA*+*nicsP*$_{lac}$::*rnhA* strain allows modulation of RNase HI. *rnhA* expression measured using qRT-PCR with Gc grown with different levels of IPTG in solid media. The average fold change compared to FA1090 (±standard deviation) for each IPTG level are: 0 IPTG-0.30 (0.09), 0.001 mM IPTG 0.61 (0.32), 0.005 mM IPTG 1.4 (0.5), 0.01 mM IPTG 4.3 (2.2), 1 mM IPTG 61.9 (33). The reported value represents the mean of five biological replicates (individual dots) +/− one standard deviation as indicated by the error bars. Unpaired student t-test . **p<0.01 *p<0.05

C. R-loop levels determined by R-loop ChIP when *rnhA* expression is modulated. The reported value represents the mean of six biological replicates (individual dots) +/− one standard deviation as indicated by the error bars. Unpaired student t-test **p<0.01

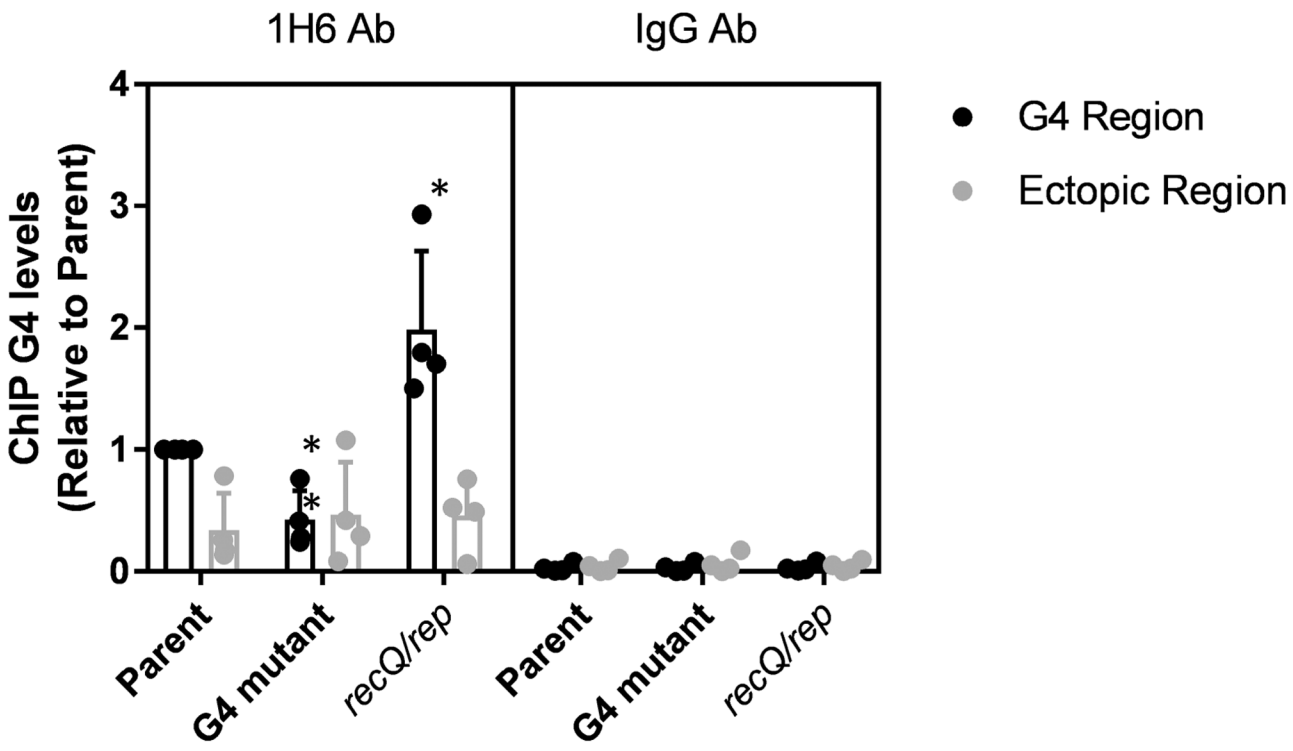

Figure 3.

G4 ChIP detects intracellular G4 levels

The sequence of the G4 motif is 5'-GGGTGGGTTGGGTGGG and G4 mutant is 5'-GTGGGGGTGTGGTGGG with 3 of 4 G-tracts interrupted. G4 levels were determined from plate grown bacteria using G4 ChIP with the 1H6 Ab. The parental strain (*recA6*) and G4 mutant, and the double helicase mutant (*recQ/rep*) were tested for G4 levels. An alternate region of the genome (ectopic site) was amplified to test for non-specific DNA affinity by the Ab. IgG was used to determine general Ab binding. The reported value represents the mean of four biological replicates (individual dots) +/− one standard deviation as indicated by the error bars. Unpaired student t-test **p<0.01 *p<0.05

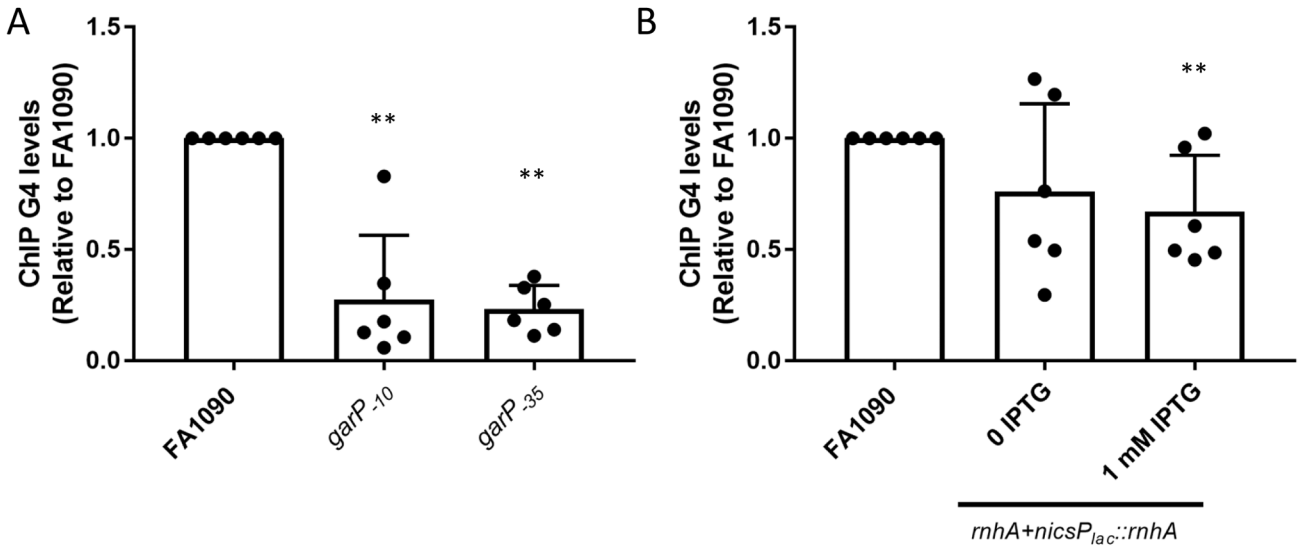

Figure 4.

Transcription initiation, but not R-loop stability, influences G4 levels

A. G4 levels were determined using G4 ChIP on *garP*$_{-10}$ and *garP*$_{-35}$ with the G4 specific MAb, 1H6). Relative G4 levels are calculated by determining the % input after ChIP pulldown for each sample and the dividing by the % input of FA1090. Bar The reported value represents the mean of six biological replicates (individual dots) +/− one standard deviation as indicated by the error bars. Unpaired student t-test **p<0.01

B. G4 ChIP was used to determine G4 levels under low *rnhA* and high *rnhA* expression (*rnhA*+*nicsP*$_{lac}$*::rnhA* 0 IPTG, and *rnhA*+*nicsP*$_{lac}$*::rnhA* 1 mM IPTG). The reported value represents the mean of six biological replicates (individual dots) +/− one standard deviation as indicated by the error bars. Unpaired student t-test**p<0.01

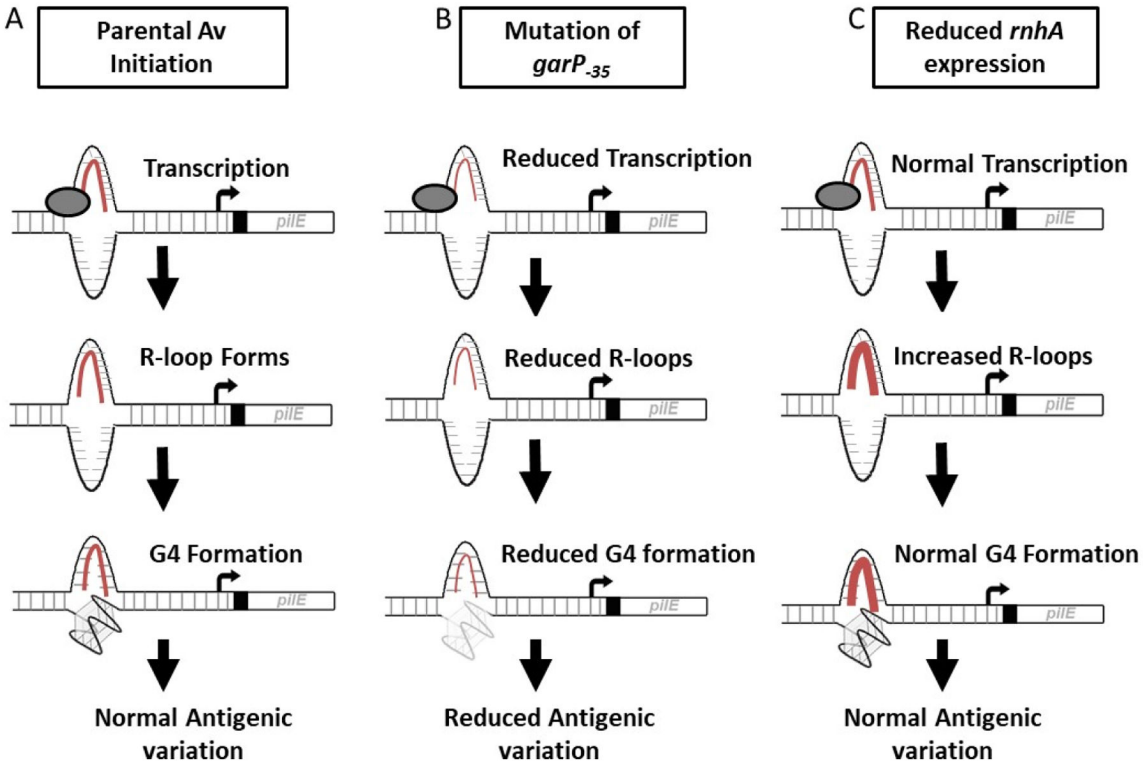

Figure 5.

Summary of results

Transcription of the G4 sRNA is the first step in Av. In A, RNA polymerase (grey oval) opens the DNA duplex. The sRNA transcript (red line) remains bound to the C rich strand of DNA, while the other DNA strand can fold into a G4 structure (grey structure with three square planes and zig zag loops). In B, reduced transcription causes reduced G4 formation and subsequent Av. In C, RNase H levels are low so R-loops are high, however, R-loop stability is dispensable for G4 levels and pilin Av.

Table 1.

Pilin Av Frequencies

Strain	% Av
FA1090	18.1
FA1090 G4 mutant	0.2
FA1090 *garP*$_{-10}$	0.1
FA1090 *garP*$_{-35}$	6.4
FA1090 *gar* 72 NT	16.9
FA1090 *gar* 32 NT	17.6
rnhA+*nicsP*$_{lac}$::*rnhA* 0 mm IPTG	10.7
rnhA+*nicsP*$_{lac}$::*rnhA* 0.005 mm IPTG	13.9
rnhA+*nicsP*$_{lac}$::*rnhA* 1 mm IPTG	13.6
rnhA+*nicsP*$_{lac}$::*rnhA garP*$_{-10}$ 0 IPTG	0.2

This table shows the combined pilin av frequencies of each condition. All variable strains were tested in biological replicates. The individual pilin Av frequencies for each barcoded pool can be found in Supplemental Table 1 along with the number of reads analyzed for each pool.