Site-specific modification of *Shigella flexneri* virF mRNA by tRNA-guanine transglycosylase *in vitro*

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ABSTRACT

*Shigella flexneri* is an enteropathogen responsible for severe dysentery in humans. VirF is a key transcriptional regulatory factor that activates the expression of the downstream virulence factors required for cellular invasion and cell-to-cell spread of this pathogen. There are several environmental facts that induce the translation of VirF including temperature, pH, osmolarity and post-transcriptional RNA modification. Durand and colleagues ([vacC](#), a virulence-associated chromosomal locus of *Shigella flexneri*, is homologous to *tgt*, a gene encoding tRNA-guanine transglycosylase of *Escherichia coli* K-12. J. Bacteriol., 176, 4627–4634) have demonstrated a correlation between VirF and tRNA-guanine transglycosylase ([TGT](#)), which catalyzes the exchange of the modified base queuine for the wobble position of the anticodon loop guanine in eukaryal and euercial tRNA (6). The proposed biochemical pathway for queuine incorporation in euercial tRNA is shown in Figure 1.

Although not yet fully understood, it is known that four genes are involved in the euercial biosynthesis of the queuine precursor, preQ1 (7). PreQ1 is incorporated into the tRNA by TGT (8). Two subsequent enzymes convert preQ1 to queuine in the tRNA (8). In contrast, euercial organisms acquire queuine from external routes such as diet, and this heterocyclic base is incorporated directly by the euercial TGT (9,10).

TGT plays a vital role in the pathogenesis of shigellosis (11), a disease that causes severe dysentery in humans. The pathogenic strain, *Shigella flexneri*, infects the cells of the human gastrointestinal tract following evasion of the host immune system defense mechanisms, such as the engulfment of foreign substances by macrophages. *Shigella* is able to escape from the macrophage endosome via the expression of certain virulence factors (Figure 2). There are several bacterial genes (including *icsA*, *IpaB*, *IpaC* and *IpaD*) that mediate this escape as well as cell-to-cell spread of the organism (12). VirF, encoded on the primary pathogenicity island of *Shigella*, is a potent transcriptional regulator of the AraC family that regulates the expression of these virulence factors (Figure 2) (13,14).

There are several environmental factors that promote the expression of VirF, including oxygen and iron limitation, temperature, pH, osmolarity and post-transcriptional RNA modification (15). Durand and Björk (11) have demonstrated a positive correlation between VirF and TGT by characterizing a mutant *Shigella flexneri* strain with an inactivated *tgt* gene (termed *vacC*). In this mutant, the translation of VirF was markedly reduced whereas the levels of *virF* mRNA

INTRODUCTION

The occurrence of non-canonical nucleosides in RNA has been well-characterized (1–4), with ~100 modified bases having been found in transfer RNA (tRNA) alone (5). Modifications occur at the post-transcriptional level, where some modifications are more simple chemical transformations (e.g. methylation) and still others are more complex (e.g. transglycosylation). One enzyme that performs a complex RNA modification (hyperm modification) is tRNA-guanine transglycosylase ([TGT](#), EC 2.4.2.29), catalyzing the exchange of the modified base queuine for the anticodon loop wobble position guanine in eukaryal and euercial tRNA (6). The proposed biochemical pathway for queuine incorporation in euercial tRNA is shown in Figure 1.

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remained unchanged, and as a result, the bacteria were unable to invade host cells. In addition, when transformed with a plasmid containing a functional Shigella tgt gene, restoring queuine modification, the Shigella mutants exhibited both restored VirF expression and virulence, thus demonstrating a positive connection between virF mRNA translation and the presence of active TGT (and presumably queuine modification).

The role of modified nucleosides in RNA structure and stability has been well-studied (16–18). Agris and Brown (19) have identified key interactions between modified nucleosides and magnesium ions essential to the secondary structure of tRNA, in addition to facilitating key RNA–protein interactions. Mandal et al. (20,21) have characterized binding of small molecules and metabolites to RNA motifs termed ‘riboswitches’. Riboswitches are structural motifs in mRNAs [sometimes in the 5′ untranslated region (UTR) and extending into the start of the open reading frame] that can exist in at least two stable conformations. One of these conformations is stabilized by binding to a small molecule, thus altering the equilibrium between the conformations. One conformation supports translation of the protein while with the other conformation, translation is blocked. In this way, binding of the small molecule changes the conformation of the RNA and modulates its translation. In addition to the 5′ UTR of prokaryotic mRNAs, riboswitches have also been found in the 3′ UTR and introns in several eukaryotic species (22). Interestingly, the Breaker lab has just reported the discovery of a riboswitch that responds to the queuine precursor, preQ1 and appears to regulate the expression of the four genes that are involved in preQ1 biosynthesis (23). It certainly seems possible that base modification of an mRNA could modulate a similar conformational change. It therefore is feasible that such a control mechanism for gene expression might be involved in regulating the expression of virulence factors in pathogenic organisms, as is apparently seen with VirF.

The incorporation of modified nucleosides has been characterized more fully for tRNA (and some other RNAs, e.g. rRNA and snRNA), than for mRNA (5,24,25). The most common example of post-transcriptional processing in mRNA is the eukaryotic 7-methylguanosine 5′ cap structure, which aids in the binding to the small ribosomal subunit and is essential for the efficient synthesis of eukaryotic proteins (26–28). To date, the only known function of TGT is to catalyze the modification of tRNA with queuine. Previous work has shown that the eubacterial TGT will recognize a U-G-U sequence in the loop of an RNA hairpin structure that corresponds to the anticodon stem–loop of its cognate tRNAs (29,30). The eubacterial TGT will also recognize a U-G-U containing hairpin in the context of a dimeric form of a cognate tRNA (31).

It is conceivable that an mRNA may also be modified directly by TGT, provided the mRNA contained the appropriate recognition elements. An examination of the sequence of virF mRNA for the presence of U-G-U sequences revealed six unique U-G-U sites (Figure 3). Mfold analysis of the regions surrounding each of these U-G-U sequences revealed that nucleotides 410–433 could possibly fold into a hairpin structure with the U-G-U sequence in a position in a loop that is analogous to the anticodon loop of TGT-cognate tRNAs.

As a first step towards probing the possibility that TGT may modulate the translation of VirF via modification of the virF mRNA, Michaelis–Menten kinetic analyses were conducted to probe this modification by TGT in vitro. We report that the Escherichia coli TGT, which has 99% sequence identity to the S. flexneri TGT, does indeed recognize the virF mRNA as a substrate in vitro. Further, we show that this recognition results in the site-specific modification of a single base in the virF mRNA.
MATERIALS AND METHODS

Reagents

Unless otherwise specified, all reagents were ordered from Sigma or Aldrich. DNA oligonucleotides, agarose, dithiothreitol (DTT), T4 DNA ligase and DNA ladders were ordered from Invitrogen. All restriction enzymes and Vent DNA polymerase were ordered from New England Biolabs. The ribonucleic acid triphosphates (NTPs) and pyrophosphatase were ordered from Roche Applied Sciences. The deoxyribonucleic acid triphosphates (dNTPs) were ordered from Promega. Low-melting Seaplaque agarose was ordered from Cambrex. Gelase™ Enzyme Prep, MasterAmp™ High Fidelity RT–PCR Kit, and Scriptguard™ RNase Inhibitor were ordered from Epicentre. Epicurian coli™ XL2-Blue ultracompetent cells were ordered from Stratagene. Amicon Ultra Centrifugal Filter Devices were ordered from Millipore. Whatman GF/C Glass Microfibre Filters and all bacterial media components were ordered from Fisher. The QIAPrep® Spin Miniprep Kit was ordered from Qiagen. Tris–HCl Buffer was ordered from Acros Organics. [3H] PreQ1 was ordered from American Radiolabeled Compounds Co.

Synthesis of [3H] preQ1 (Figure 4)

The cyano precursor (preQ0, 2) was synthesized according to the method of Migawa et al. (33) by the condensation of chloro(formyl)acetonitrile and pyrimidine 1 (33). Reduction of the cyano precursor with tritium gas gave the desired radiolabeled substrate preQ1 (3) with a specific activity of 500 mCi/mmol (34). The tritium reduction was performed commercially by American Radiolabeled Compounds Co.

Construction of pTZvirF

The plasmid pBDG302, containing the virF gene, was received from Prof. Glenn Bjoerk (Umeå University, Sweden). The virF gene was amplified from the plasmid by polymerase chain reaction (PCR) under the following conditions: primers (20 pmol each), pBDG302 (500 ng), Mg2+ (2 mM), dNTPs (1 mM each), Vent DNA
polymerase (4 U), brought to a final volume of 50 μl with deionized water. The sample was treated with 30 PCR cycles of the following sequence: 94°C (1 min), 50°C (1 min), and 72°C (2 min), followed by a final extension at 72°C (5 min). Following a double restriction enzyme digest with PstI and EcoRI (40 U each, 20-μl reaction) for 1 h at 37°C, the PCR product and vector were gel-purified from Seaplaque agarose with Gelase™ according to the vendor protocol. The purified virF gene was then ligated into digested pTZ19R^Amp (5:1 volume ratio, 20 μl reaction) following overnight incubation with T4 DNA ligase (2 U) at 17°C. The ligated sample (10 μl) was transformed into 100 μl of Epicurian coli® XL2-Blue ultracompent cells according to the Stratagene protocol. Cells were grown overnight at 37°C on L-Amp plates (50 μg/ml ampicillin). Individual colonies were isolated, and 3 mL 2xTY (16 g Bactotryptone, 10 g yeast extract, 5 g NaCl/liter of water with 50 μg/ml ampicillin) liquid cultures were inoculated at 37°C with shaking. Plasmid was isolated via miniprep, and the virF gene sequence was confirmed with DNA sequencing (University of Michigan DNA Sequencing Core Facilities).

**In vitro transcription**

*In vitro* transcription reactions with pTZvirF were conducted by first linearizing the plasmid at the end of the virF sequence with the restriction enzyme EcoRI (40 U/100 μl DNA, 500 μl reaction). The sample was ethanol precipitated at −20°C, and the pellet was re-suspended in 250 μl of deionized water. *In vitro* 1 ml transcription conditions were as follows: pTZvirF template (100 μl), transcription buffer (4 mM Tris–HCl, pH 8.0; 2 mM MgCl₂, 0.5 mM DTT, 0.1 mM spermidine), NTPs (4 mM each), T7 RNA polymerase (2500 U), inorganic pyrophosphatase (2 U) and RNase inhibitor (200 U). The reaction was incubated at 37°C for ~4 h. The reaction was stored at −20°C following transcription. Best results were obtained when the 1 ml reaction was prepared when the 1 ml reaction was prepared and redistributed into 100 μl volumes prior to incubation at 37°C. The MasterAmp™ High Fidelity RT–PCR Kit was used according to vendor protocol to generate virF DNA, which was confirmed with sequence analysis of the DNA product (Figure 5).

**Mfold analysis and synthesis of virF MH**

Analysis of the energetically favorable secondary structures within the virF mRNA sequence was performed using the biophysical web tool Mfold (M. Zuker, http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). Sequences of ~10 nucleotides surrounding either side of the six possible recognition motifs were analyzed by the web tool, and the hairpin structure determined to be most favorable was found between nucleotides 410–433 in the virF mRNA, which contains the potential recognition sequence U₄₂₀G₄₂₁U₄₂₂. The RNAture Oligonucleotide Analyzer web tool was used to predict a Tm of 71°C for the virF minihelix hairpin. (Note: This web tool appears to be no longer available.) An Expedite™ Nucleic Acid Synthesis System was used to synthesize this 24-nucleotide sequence (5’-GGAGAGAUGCCUUUGUCGACUAAUUUU-3’) using the vendor’s protocols for the synthesis of RNA at the 1 μmol scale. The reagents were from Perkin–Elmer and the RNA amidites were from Glen Research. The extinction coefficient calculated for this RNA minihelix was ⨁₂₅₀ = 265.3 OD/MM.

**Generation of virF mRNA(G₄₂₁A)**

The single nucleotide mutation of guanine 421 to adenine in the virF mRNA sequence was generated via QuikChange site-directed mutagenesis (Stratagene), producing the new vector pTZvirF(G₄₂₁A). The reactions conditions were as follows: complimentary oligonucleotides with desired mutation (175 ng), pTZvirF(wt) template (800 ng), dNTPs (0.25 mM) and Vent DNA polymerase (2 U), brought to a final volume of 30 μl with deionized water. The sample was treated with 25 PCR cycles of the following sequence: 94°C (30 s), 50°C (1 min), and 72°C (6.5 min). The PCR product was then incubated for 2 h at 37°C with Dpn I (40 U), and addition of NE Buffer 4 was required for proper digestion of wild-type plasmid. The digested sample (10 μl) was transformed into 100 μl of Epicurian coli® XL2-Blue ultracompent cells according to the Stratagene protocol. Cells were grown overnight at 37°C on L-Amp plates (50 μg/ml ampicillin). Individual colonies were isolated, and 3 ml 2xTY (16 g bactotryptone, 10 g yeast extract, 5 g NaCl/liter of water with 50 μg/ml ampicillin) liquid cultures were inoculated at 37°C with shaking. Plasmid was isolated via miniprep, and the virF mRNA(G₄₂₁A) mutation was confirmed with DNA sequencing (University of Michigan DNA Sequencing Core Facilities).

**Kinetic assays**

Assays were conducted by monitoring the incorporation of radiolabeled substrate, [³H] preQ₁, into E. coli tRNA^Thr^, dG₃₁ECYMH (5’-GGGAGACGACUdGUA AUCUGCUCC-3’) and various virF mRNA substrates. Samples from *in vitro* transcriptions were concentrated...
RESULTS

Construction and in vitro transcription of pTZvirF

To provide micromolar quantities of virF mRNA for our studies, we generated an in vitro transcription clone for the virF mRNA. The virF gene was subcloned from the plasmid pBDG302 containing the virF gene (a gift from Professor Glenn Björk, Umeå University, Sweden) into a plasmid suitable for in vitro transcription, generating pTZvirF. VirF mRNA was synthesized via in vitro run-off transcription following digestion with EcoRI, linearizing pTZvirF at the end of the virF gene sequence. The virF mRNA was physically characterized on an ethidium bromide stained, 12% formaldehyde agarose gel. RT-PCR was utilized to generate dsDNA from the in vitro transcription product using the same oligonucleotide primers initially designed for subcloning of the virF gene. Examples of formaldehyde and TAE agarose gels of the virF mRNA and the dsDNA from the RT–PCR are shown in Figure 5.

The single-stranded virF mRNA appears to run on the gel at ~500bp in comparison to the dsDNA ladder. The size of the virF gene is 789bp, and the corresponding mRNA is 789 nucleotides in length. We hypothesize that the mRNA is running at a lower ‘apparent’ molecular weight due to the propensity of mRNA to adopt a variety of conformations, even in an agarose gel. This would explain why the observed molecular weight is a little larger than one half the size of the double-stranded virF DNA.

Kinetic analysis of E. coli tRNA^Tyr, ECYMH minihelix with preQ1

For comparison, Michaelis–Menten kinetic analyses were conducted with the natural RNA substrate E. coli tRNA^Tyr (ECY) and the modified minihelix substrate dG34ECYMH (the anticodon stem–loop of ECY where the guanosine at position 34 contains a 2′-deoxyribose) with [3H]preQ1. It has been shown previously that a minihelix RNA consisting of the anticodon arm and loop of a queueine-cognate tRNA is a sufficient substrate for TGT (29). Aliquots were taken at various time points over a 15 min incubation of 100 nM E. coli TGT, various concentrations of ECY (0.05–1.5 μM) or dG34ECYMH (0.05–5 μM) and saturating concentrations of [3H]preQ1. The kinetic constants determined for the incorporation of [3H]preQ1 with ECY and dG34ECYMH are shown in comparison with the kinetic data for the virF substrates in Table 1.

Kinetic analysis of virF mRNA, virF MH minihelix with preQ1

Using the same approach described above, Michaelis–Menten kinetic analyses were conducted with virF mRNA. Aliquots were taken at various time points over a 1 h incubation of 200 nM TGT, various concentrations of virF mRNA (0.1–10 μM), and saturating concentrations of [3H]preQ1 (Figure 6A). Higher concentrations of virF mRNA were tested to obtain an accurate kinetic profile by characterizing the reaction over a large range of concentrations. In addition to characterizing the wild-type virF mRNA, a virF minihelix RNA (virF MH) corresponding to the 410–433 hairpin sequence (underlined in Figure 3) as well as a full-length virF mRNA mutant (G421A) were studied. The kinetic analyses were performed with 100 nM E. coli TGT, various concentrations of virF MH (0.1–10 μM) and saturating concentrations of [3H]preQ1 (Figure 6B). The full-length virF mRNA(G421A) was incubated under the same conditions as the wild-type mRNA, but only at concentrations corresponding to KM 5x KM, as determined from the kinetic constants of virF mRNA(wt) (Table 1). A ‘no RNA’ control was also included to determine the background level of radioactivity present in the samples (Figure 6C). The data were fit by non-linear regression.

Both the full-length virF mRNA(wt) and the virF minihelix exhibited RNA concentration-dependent incorporation of [3H]preQ1 over time, and the Michaelis–Menten equation provided a good fit for the data. The full-length virF mRNA(G421A), which is
full-length mRNA with a single nucleotide mutation at guanine 421, was analyzed at both 2 μM and 10 μM mRNA. The virF mRNA(G421A) mutant showed no detectable activity greater than the ‘no RNA’ control (Figure 6C). The kinetic constants determined for the virF mRNA substrates are shown in Table 1.

Both the virF mRNA and virF MH have K_M values in the low micromolar range, even though k_cat and k_cat/K_M for both is lower than the corresponding values for the ECY substrates.

**DISCUSSION**

VirF is a critical transcriptional regulator responsible for activating virulence genes in *Shigella flexneri*. Durand and colleagues (35) demonstrated the involvement of TGT in modulating the translation of VirF via the observation...
that a mutant strain of Shigella with an inactivated \( \text{tng} \) gene (termed \( \text{vacC} \)) showed decreased virulence. VirF protein levels were dramatically lower in the mutant as compared to wild-type, but the \( \text{virF} \) mRNA levels showed no detectable difference from wild-type Shigella. The lack of VirF protein resulted in a reduction of all downstream virulence gene expression, and thus exhibited a less virulent phenotype than that of the wild-type bacterium.

When transformed with a plasmid encoding the Shigella \( \text{tng} \) gene, both VirF translation and virulence were restored. It had previously been shown that the presence of modified nucleosides enhances translation (36,37). However, other studies have shown that growth rate and protein translation as a whole are not directly affected by a lack of queuine-modified tRNA (38). While this interesting correlation between VirF translation and TGT activity has been known for some time, yet the exact role TGT plays in the translation of this primary virulence factor remains unclear.

Our laboratory has previously demonstrated that TGT can modify substrates with more unusual structures than a canonical tRNA fold. We reported that a dimeric form of the ECY serves as a substrate for TGT, with a slightly higher \( K_M \) and identical \( k_{cat} \), relative to the normal tRNA (31). It had previously been shown from NMR studies that the anticodon arms of the dimer subunits were intact and pointing away from the center of the dimer (39).

Those studies demonstrate that TGT can recognize a minihelix containing the requisite U-G-U sequence even in the context of a larger RNA structure. TGT is not the first tRNA modification enzyme to demonstrate recognition of alternative RNA structures. Gu and coworkers (24) have shown that, \textit{in vitro}, the modification enzyme tRNA (\( m^{\text{U54}} \))-methyltransferase will methylate 16S ribosomal RNA from \( \text{E. coli} \) in addition to its physiological tRNA (38). This interesting correlation between VirF translation and TGT activity has been known for some time, yet the exact role TGT plays in the translation of this primary virulence factor remains unclear.

Our analysis of the \( \text{virF} \) mRNA sequence predicts that there should be a single site of modification, guanine 421. We have taken two approaches to investigate this. In our first approach, a \( \text{virF} \) MH corresponding to the predicted hairpin structure within the native \( \text{virF} \) mRNA sequence (bases 410–433), was chemically synthesized (Figure 6B). The stem consists of nine base pairs, where the first four nucleotides in the stem are uridine residues forming wobble pair interactions with three guanines and one Watson–Crick pair with an adenosine. At first glance, the stability of a minihelix with three G-U wobble pairs might be questionable; however, in the context of the \( \text{virF} \) mRNA, the ends of the helix may be held in close proximity by other intramolecular interactions. Additionally, the \( \text{virF} \) MH by itself has a predicted melting temperature of 71°C, indicating the structure should be stable at physiological and assay temperatures. The \( \text{virF} \) MH is a substrate for TGT \textit{in vitro}. The \( K_M \) values for both the full-length \( \text{virF} \) mRNA and \( \text{virF} \) MH (1.8 and 0.87 \( \mu \text{M} \), respectively; Table 1) are very similar, suggesting that the minihelix structure is likely a predominant conformation in the \( \text{virF} \) mRNA.

Although the recognition of the \( \text{virF} \) minihelix by TGT is consistent with the \( \text{virF} \) mRNA serving as a substrate for TGT, it does not provide conclusive evidence that guanine 421 is the site of modification in the \( \text{virF} \) mRNA. There are six UGU sequences within the \( \text{virF} \) mRNA that are possible recognition sites for TGT. Therefore, our second approach was to construct the point mutation, G421A, in the full-length \( \text{virF} \) mRNA to demonstrate the importance of guanine 421. The \( \text{virF} \) mRNA(G421A)
mutant resulted in a complete loss of activity at two different concentrations of RNA (Figure 6C), indicating that G421 is indeed essential for recognition by TGT. Had a second exchangeable guanine existed in the sequence, we would have expected to see a decreased or possibly even unchanged activity of the mRNA. The relationship between the ‘no RNA’ negative control and the virF mRNA(G421A) indicates that guanine 421 is the only exchangeable nucleotide in the virF mRNA sequence (at least within the concentration ranges tested), and that the kinetic parameters observed for virF mRNA(wt) are due to specific recognition by TGT and could not be attributed to non-specific interactions with this large nucleic acid molecule. It should be noted that, under the conditions of the assay (Figure 6C), it appears that the enzyme is undergoing a limited number of turnovers. Two factors may be contributing to this. The first is that our calculations of kinetic parameters assume 100% active enzyme, which is almost certainly an over-estimate. The active enzyme concentration may be as much as 2-fold lower as recent studies suggest that the eu-bacterial TGT may exist as a homodimer with ‘half-of-sites’ reactivity (49). This would effectively double the turnovers per active site. Second, the off-rate for the modified mRNA may be sufficiently slower (relative to that for tRNA) such that the turnover rate may indeed be significantly slowed under these conditions. It remains to be seen if these observations hold under in vivo conditions.

From the results presented herein, it is clear that the virF mRNA does act as a substrate for the eu-bacterial TGT in vitro. Although there are six possible UGU recognition motifs, both the mutagenesis and virF mRNA minihelix studies are consistent with G421 serving as the sole site of modification within the mRNA. With a \( K_M \) value in the low micromolar range, it is very possible that the modification of virF mRNA may be biologically relevant (e.g. may occur in vivo). These results provide the first ‘proof of principle’ evidence that post-transcriptional RNA modification may regulate RNA function, as it has long been recognized to do for tRNA. The recent work characterizing the preQ1 riboswitch revealed that it is a fairly simple hairpin structure (the simplest riboswitch structure characterized to date) (23). Such a simple structure could feasibly occur in the coding region of an mRNA species. In fact, the TGT modification site that we have discovered in the virF mRNA is predicted to occur in a simple hairpin structural motif. Base modification in a hairpin structure in the coding sequence of virF mRNA could induce a similar structural switch as seen in the preQ1 riboswitch and thereby influence translation of VirF. Studies to determine the physiological significance of the virF mRNA modification by TGT that we have observed in vitro are currently in progress.

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