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 regulator 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 factors 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 hypermodified base queuine for the guanine in the wobble position of certain tRNAs. They characterized *tgt*- mutant *S. flexneri* strains in which the translation of VirF is markedly reduced and the bacteria are unable to invade host cells. Although the function of TGT is to modify tRNA, we report that the *virF* mRNA is recognized by the *Escherichia coli* TGT (99% identity to the *S. flexneri* TGT) *in vitro*. Further, we show that this recognition results in the site-specific modification of a single base in the *virF* mRNA. In the context of previous reports that small molecule binding motifs ('riboswitches') in mRNAs modulate mRNA conformation and translation, our observations suggest that TGT may modulate the translation of VirF by base modification of the VirF encoding 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 (hypermodification) 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 eukaryl and eubacterial tRNA$^{Asp, Asn, His, Tyr}$ (6). The proposed biochemical pathway for queuine incorporation in eubacterial tRNA is shown in Figure 1.

Although not yet fully understood, it is known that four genes are involved in the eubacterial biosynthesis of the queuine precursor, preQ$_1$ (7). PreQ$_1$ is incorporated into the tRNA by TGT (8). Two subsequent enzymes convert preQ$_1$ to queuine in the tRNA (8). In contrast, eukaryl organisms acquire queuine from external routes such as diet, and this heterocyclic base is incorporated directly by the eukaryl 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
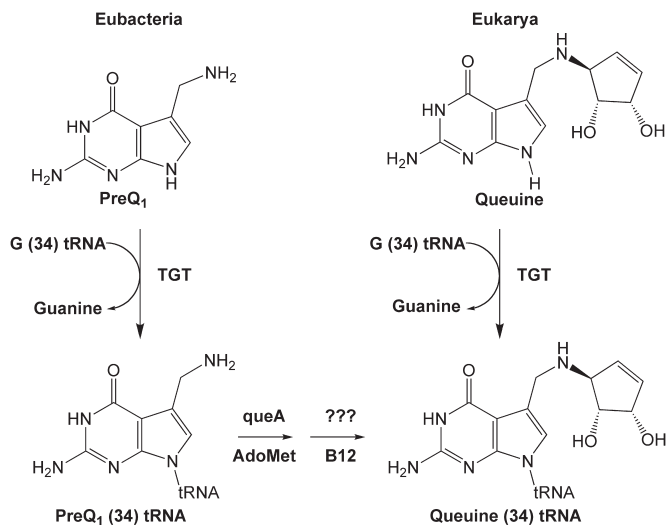
Figure 1. Proposed biochemical pathway for queuine incorporation in eubacterial and eukaryl tRNA.
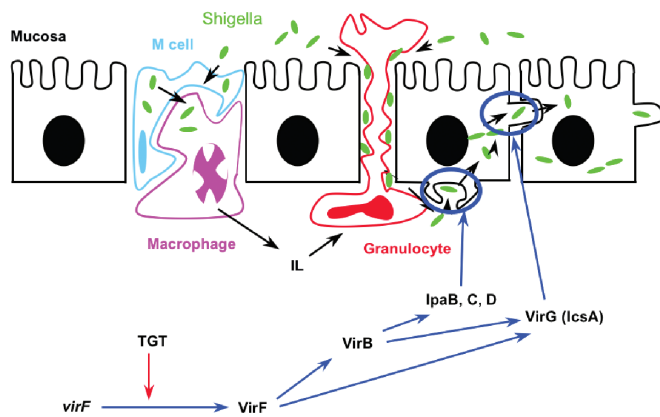
Figure 2. Schematic representation of *Shigella* pathogenesis. The invasion of the intestinal mucosal cells by *S. flexneri* is regulated by a number of virulence factors, including the transcriptional regulator, VirF. The VirF protein then activates the transcription of downstream virulence factors under the proper environmental conditions for invasion. Durand and colleagues (35) have shown that TGT is linked to the efficient translation of the VirF protein. Figure adapted from one provided by Prof. Ruth Brenk, University of Dundee.

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, preQ$_1$ and appears to regulate the expression of the four genes that are involved in preQ$_1$ 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 reveal 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.

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VirF

aug aug gau aug gga cau aaa aac aaa aua gau aua aag guu cgc uug cau aac uau auu auu uua uau gca
 M   M   D   M   G   H   K   N   K   I   D   I   K   V   R   L   H   N   Y   I   I   L   Y   A

aaa agg UGU uca aug acg guu agc uca ggc aau gaa acu uug acu auc gau gaa ggg caa auu gcu uuu aua
 K   R   C   S   M   T   V   S   S   G   N   E   T   L   T   I   D   E   G   Q   I   A   F   I

gag cga aau aua caa aua aac guc ucc aua aaa aaa ucu gau agc aua aau cca uuu gag auu aua agc cuu
 E   R   N   I   Q   I   N   V   S   I   K   K   S   D   S   I   N   P   F   E   I   I   S   L

gac aga aau uua uua uua agc auu auu aga aua aug gaa cca auu uau uca uuu caa cac ucc uau ucu gag
 D   R   N   L   L   L   S   I   I   R   I   M   E   P   I   Y   S   F   Q   H   S   Y   S   E

gag aaa agg ggg uua aac aaa aaa aua uuc cuc cuc ucu gag gag gag guu ucu auc gau uUG Uuc aaa ucu
 E   K   R   G   L   N   K   K   I   F   L   L   S   E   E   E   V   S   I   D   L   F   K   S

aua aaa gag aug ccu uuc ggc aaa aga aag auc uau agu uua gcu ugc cuu uua uca gcU GUu ucu gau gag
 I   K   E   M   P   F   G   K   R   K   I   Y   S   L   A   C   L   L   S   A   V   S   D   E

gaa gcu uua uau acu ucg aua ucg aua gcu ucu ucu cuu agu uuu ucu gau cag aua agg aag auU GUu gaa
 E   A   L   Y   T   S   I   S   I   A   S   S   L   S   F   S   D   Q   I   R   K   I   V   E

aaa aac auc gag aag aga ugg cgu cuu ucu gau auu uca aau aac uug aau uua uca gaa aua gcU GUu aga
 K   N   I   E   K   R   W   R   L   S   D   I   S   N   N   L   N   L   S   E   I   A   V   R

aaa cga uug gag agu gaa aaa uua aca uuu caa caa auc cuu uua gau auu cgc aug cau cau gca gca aag
 K   R   L   E   S   E   K   L   T   F   Q   Q   I   L   L   D   I   R   M   H   H   A   A   K

cuu uua uug aau agu caa agc uau auu aau gaU GUa uca aga cuu auc gga aua uca agc cca ucu uau uuu
 L   L   L   N   S   Q   S   Y   I   N   D   V   S   R   L   I   G   I   S   S   P   S   Y   F

aua agg aaa uuu aau gaa uau uau ggu aua acu cca aag aaa uuu uac uua uau cau aaa aaa uuu uaa
 I   R   K   F   N   E   Y   Y   G   I   T   P   K   K   F   Y   L   Y   H   K   K   F   *
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Figure 3. Nucleotide sequence of *virF* mRNA. Six UGU sequences are found in the sequence, and are highlighted in red. The sequence of the *virF* MH is underlined.

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 Chemicals, Inc. T7 RNA polymerase was isolated from *E. coli* BL21 cells containing the plasmid pBH161 according to the procedure of Prof. William McCallister, State University of New York, Brooklyn. The *E. coli* TGT was isolated with an amino terminal his-tag as previously described (32).
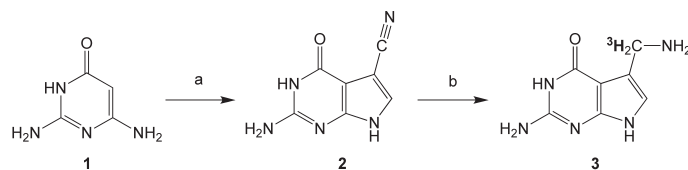

Figure 4. Synthesis of radiolabeled [³H] preQ₁. (**A**) chloro(formyl) acetonitrile, NaOAc, H_2O, 100°C; (**B**) ³H₂, Pd/C, MeOH, 50 psi.

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 Björk (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), Mg^{2+} (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 ultracompetent 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 resuspended 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 MgCl$_2$, 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 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 U$_{420}$G$_{421}$U$_{422}$. 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′-GGAGUAGUCUUUGUCGACUAUUUU-3′) using
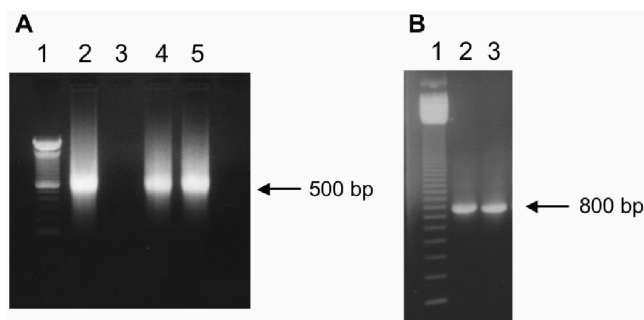
Figure 5. Transcription of *virF* mRNA. (**A**) *In vitro* transcription of *virF* mRNA visualized with ethidium bromide on a 1% TAE agarose gel. The single-stranded mRNA ran at ∼500 bp in comparison to the 100-bp DNA ladder (lane 1). Lane 2: *virF* mRNA from a previous transcription reaction. Lanes 4 and 5: *virF* mRNA from replicate transcription reactions. (**B**) RT–PCR samples visualized with ethidium bromide on a 1% TAE agarose gel. RT–PCR was conducted using extracted mRNA from replicate samples (lanes 2 and 3), electrophoresed in comparison to a 100 bp DNA ladder (lane 1). Each RNA sample produced the corresponding 800 bp DNA species indicative of the *virf* ORF.

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 ε$_{260}$ = 265.3 OD/mM.

Generation of *virF* mRNA(G$_{421}$A)

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(G$_{421}$A). 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 ultracompetent 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(G$_{421}$A) 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, [^{3}H] preQ$_1$, into *E. coli* tRNA^{Tyr}, dG$_{34}$ECYMH (5′-GGGAGCAGACUdGUAA AUCUGCUCCC-3′) and various *virF* mRNA substrates. Samples from *in vitro* transcriptions were concentrated

with Amicon Ultra Centrifugal Filters (10,000 MWCO). The concentration of *virF* mRNA was determined with a Cary UV-Visible Spectrophotometer, where the approximate concentration of a single-stranded RNA sample $A_{260} = 1$ OD is $40\,\mu g/ml$ and the molecular weight of *virF* mRNA is $252\,g/mmol$ *virF* mRNA. In brief, kinetic assays were set up under the following conditions: RNA substrate (various concentrations), [^{3}H] preQ$_1$ ($10\,\mu M$, 296mCi/mmol stock), TGT (100 or 200 nM, as specified), and HEPES reaction buffer (100 mM HEPES, pH 7.3; 20 mM MgCl$_2$; 5 mM DTT) to a final volume of $400\,\mu l$. All samples were incubated at $37°C$ for purposes of equilibration before initiating the reaction with the addition of TGT. Aliquots ($70\,\mu l$) were removed at various points throughout the reaction and quenched in 3 ml of 5% TCA for 1 h before filtering on glass-fiber filters. Each filter was washed with three volumes of 5% TCA and a final wash of ethanol to dry the filter. The samples were analyzed in a scintillation counter (Beckman) for radioactive decay, where counts were reported in DPM and later converted to picomoles [^{3}H] preQ$_1$ by the following conversion (pmol = DPM × 0.00152, for the [^{3}H] preQ$_1$ stock with a specific activity of 296 mCi/mmol). Initial velocities were determined by converting the slopes of these plots (pmol/min) to units of second^{-1}, taking into account the concentration of the enzyme and aliquot size. The individual data points from each trial were averaged, and the standard deviation was determined for each concentration of RNA substrate. The average data points (with error bars representing their standard deviations) were plotted. However, all of the individual data points were fit via non-linear regression to the Michaelis–Menten equation and the line for that fit is displayed (Figure 6). All non-linear regression fits with the Michaelis–Menten equation were determined using Kaleidagraph (Abelbeck Software).

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, 1.2% 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 ~500 bp in comparison to the dsDNA ladder. The size of the *virf* gene is 789 bp, 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. The RT–PCR product (dsDNA) migrated to the anticipated molecular weight for a DNA sample of ~800 bp. DNA sequencing of this product matched the *virF* mRNA sequence (data not shown).

Kinetic analysis of *E. coli* tRNA^{Tyr}, ECYMH minihelix with preQ$_1$

For comparison, Michaelis–Menten kinetic analyses were conducted with the natural RNA substrate *E. coli* tRNA^{Tyr} (ECY) and the modified minihelix substrate dG$_{34}$ECYMH (the anticodon stem–loop of ECY where the guanosine at position 34 contains a 2′-deoxyribose) with [^{3}H] preQ$_1$. It has been shown previously that a minihelix RNA consisting of the anticodon arm and loop of a queuine-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 ($0.05–1.5\,\mu M$) or dG$_{34}$ECYMH ($0.05–5\,\mu M$) and saturating concentrations of [^{3}H] preQ$_1$. The kinetic constants determined for the incorporation of [^{3}H] preQ$_1$ with ECY and dG$_{34}$ECYMH are shown in comparison with the kinetic data for the *virF* substrates in Table 1.

Kinetic analysis of *virF* mRNA, *virF* MH minihelix with preQ$_1$

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 ($0.1–10\,\mu M$), and saturating concentrations of [^{3}H] preQ$_1$ (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 (G$_{421}$A) were studied. The kinetic analyses were performed with 100 nM *E. coli* TGT, various concentrations of *virF* MH ($0.1–10\,\mu M$) and saturating concentrations of [^{3}H] preQ$_1$ (Figure 6B). The full-length *virF* mRNA(G$_{421}$A) was incubated under the same conditions as the wild-type mRNA, but only at concentrations corresponding to K_M and 5xK_M, 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 [^{3}H] preQ$_1$ over time, and the Michaelis–Menten equation provided a good fit for the data. The full-length *virF* mRNA(G$_{421}$A), which is
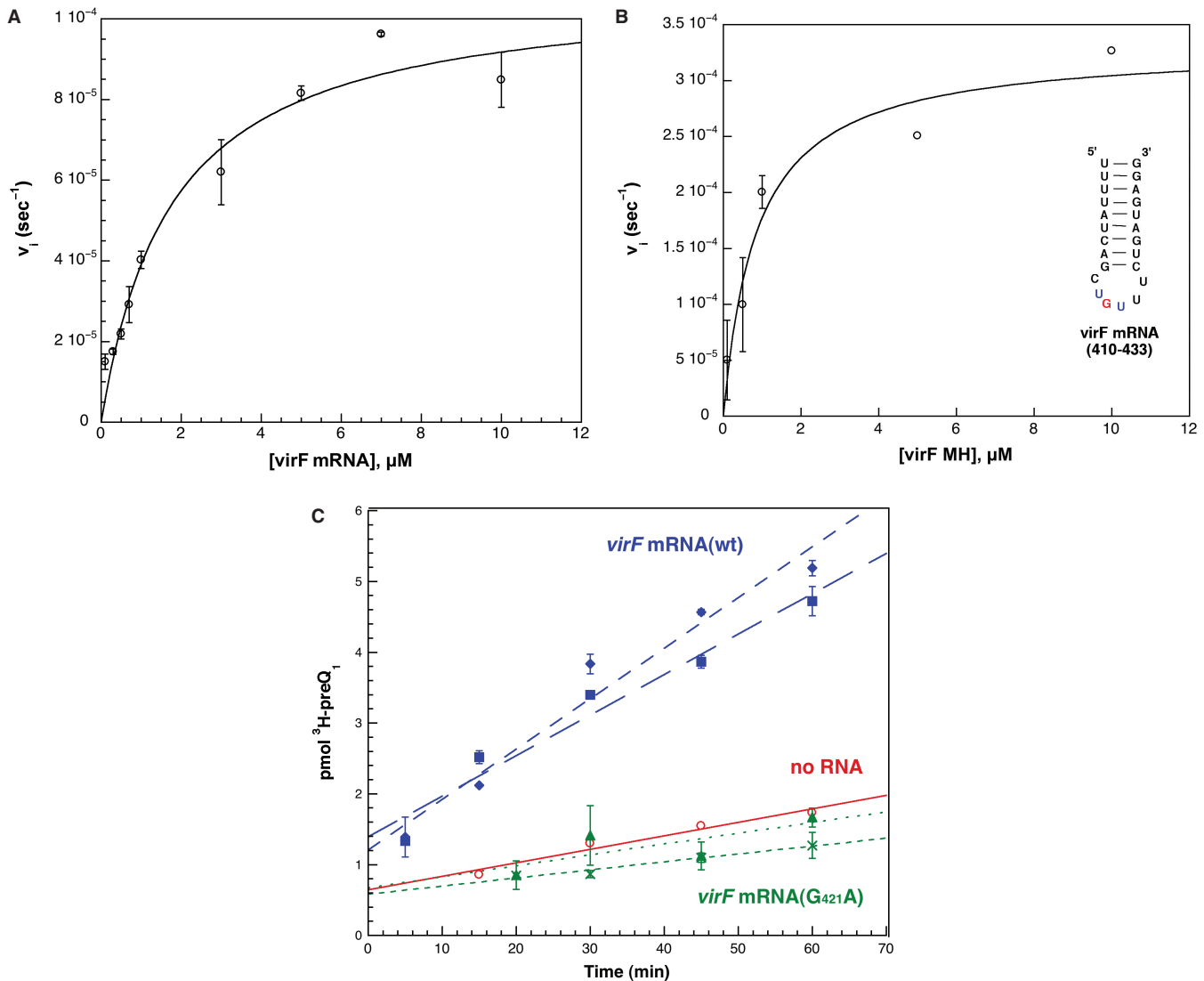
Figure 6. Kinetic characterization of *virF* mRNA substrates with *E. coli* TGT and [³H] preQ₁. (**A**) and (**B**) The individual data points were fit to the Michaelis–Menten equation by non-linear regression, and the average data points (with error bars representing the standard deviations of the averages) for each concentration of RNA substrate are depicted. The study was performed with duplicate kinetic trials at saturating concentrations of [³H] preQ₁. The inset in B shows the structure of the RNA minihelix corresponding to bases 410–433 of the *virF* mRNA. (**C**) The comparison of radioactive substrate incorporated over time in *virF* mRNA(wt) (blue, squares and diamonds), *virF* mRNA(G₄₂₁A) (green, crosses and triangles), and a no RNA control (red, circles), fit by linear regression. Each *virF* mRNA substrate was analyzed at two concentrations: 2 µM (squares and crosses) and 10 µM (diamonds and triangles). The average of duplicate trials is shown.

Table 1. RNA kinetic parameters with *E. coli* htTGT(wt) and [³H] preQ1 at pH 7.3

	K_M (µM)	k_{cat} (10^{-4} s^{-1})	k_{cat}/K_M (10^2 M^{-1} s^{-1})
ECY	0.16 (0.03)[a]	40 (2)	250 (40)
dG₃₄ECYMH	0.26 (0.07)	70 (5)	270 (60)
virF mRNA	1.8 (0.4)	1.1 (0.1)	0.60 (0.1)
virF MH	0.87 (0.3)	3.3 (0.3)	3.8 (1)

[a]Standard errors are in parentheses.

full-length mRNA with a single nucleotide mutation at guanine 421, was analyzed at both 2 µM ($\sim K_M$) and 10 µM ($\sim 5 \times K_M$) 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 *tgt* gene (termed *vacC)* showed decreased virulence. VirF protein levels were dramatically lower in the mutant as compared to wild-type, but the *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 tgt* 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, *in vitro*, the modification enzyme tRNA (m^5U54)-methyltransferase will methylate 16S ribosomal RNA from *E. coli* in addition to its physiological tRNA substrate, although they found no evidence for this occurring *in vivo*. The enzyme pseudouridine synthase catalyzes the isomerization of specific uridines to the modified nucleoside ψ. A single pseudouridine synthase has been shown to have 'dual specificity', recognizing and modifying both tRNA and snRNA (40–42). Ofengand and colleagues (43,44) have also characterized a critical pseudouridine synthase responsible for site-specific modification with pseudouridine *in vivo* for both tRNA and 23S rRNA in *E. coli*. Such precedence for tRNA modification enzymes to recognize and modify other RNA species *in vitro* and *in vivo*, suggests that *virF* mRNA modification mediated by TGT may also occur. As a first step to probe for this possibility, we examined the *virF* mRNA sequence for the presence of a U-G-U sequence in a TGT recognition motif. Of the six U-G-U sequences in the *virF* mRNA, the one involving bases 410–433 (Figure 3) was predicted by Mfold analysis to be able to fold into a hairpin structure possibly suitable for recognition by TGT.

Incubation of *virF* mRNA with *E. coli* TGT and radiolabeled preQ$_1$ revealed that the *virF* mRNA is indeed a substrate for TGT *in vitro*. The kinetic parameters (K_M, k_{cat}; Table 1) for *virF* mRNA were determined from a Michaelis–Menten analysis (Figure 6A). The *virF* mRNA substrates exhibit the same trend in kinetic parameters as

the ECY substrates, where the minihelix substrates (dG$_{34}$ECYMH and *virF* MH) have slightly higher values for both K_M and k_{cat}/K_M with respect to the corresponding full-length RNA substrates (Table 1). The values of K_M for both ECY and *virF* mRNA are very similar, both in the low micromolar range. This is encouraging considering the size and structural difference between the two substrates, where ECY is ~80 nucleotides in length with a very well-defined tertiary structure common to tRNA and the *virF* mRNA is *ca.* 800 nucleotides in length and presumably does not have a compact tertiary structure. The Michaelis–Menten plot for the *virF* mRNA (Figure 6A) fits to a single K_M, consistent with a single site of modification within the *virF* mRNA. The k_{cat} for the *virF* mRNA is ~ 40-fold lower than that for tRNA. It has previously been shown that altering the position of the U-G-U sequence within the minihelix loop of cognate tRNAs is correlated with a reduction in activity (45). All of the biochemical and structural data previously reported is consistent with a covalent intermediate, via Asp264, in the TGT reaction (32,46–48). The hairpin loop of the ECY substrate contains seven nucleotides, whereas the hairpin in the *virF* mRNA substrate contains only six nucleotides in the loop. This difference in loop length may result in a suboptimal orientation of the guanosine ribose in the U$_{420}$-G$_{421}$-U$_{422}$ loop in the *virF* mRNA, making nucleophilic attack by Asp264 less likely. This difference in loop length and orientation could account for the reduced k_{cat} that we have observed for the *virF* mRNA substrates.

Our analysis of the *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 *virF* MH corresponding to the predicted hairpin structure within the native *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 *virF* mRNA, the ends of the helix may be held in close proximity by other intramolecular interactions. Additionally, the *virF* MH by itself has a predicted melting temperature of 71°C, indicating the structure should be stable at physiological and assay temperatures. The *virF* MH is a substrate for TGT *in vitro*. The K_M values for both the full-length *virF* mRNA and *virF* MH (1.8 and 0.87 μM, respectively; Table 1) are very similar, suggesting that the minihelix structure is likely a predominant conformation in the *virF* mRNA.

Although the recognition of the *virF* minihelix by TGT is consistent with the *virF* mRNA serving as a substrate for TGT, it does not provide conclusive evidence that guanine 421 is the site of modification in the *virF* mRNA. There are six UGU sequences within the *virF* mRNA that are possible recognition sites for TGT. Therefore, our second approach was to construct the point mutation, G$_{421}$A, in the full-length *virF* mRNA to demonstrate the importance of guanine 421. The *virF* mRNA(G$_{421}$A)

mutant resulted in a complete loss of activity at two different concentrations of RNA (Figure 6C), indicating that G_{421} 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(G_{421}A) 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 eubacterial 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 eubacterial TGT *in vitro*. Although there are six possible UGU recognition motifs, both the mutagenesis and *virF* mRNA minihelix studies are consistent with G_{421} 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 mRNA function, as it has long been recognized to do for tRNA. The recent work characterizing the preQ$_1$ 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 preQ$_1$ 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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