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Research Article

Silencing Transcription from an Influenza Reverse Genetics Plasmid in *E. coli* Enhances Gene Stability

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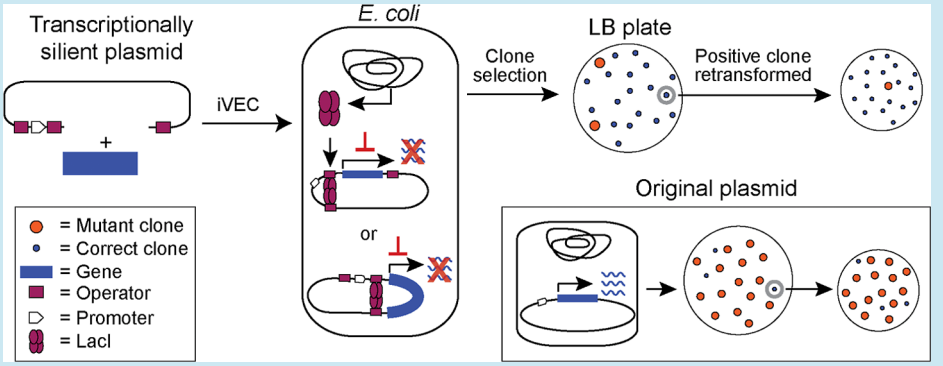

ABSTRACT: Reverse genetics (RG) systems have been instrumental for determining the molecular aspects of viral replication, pathogenesis, and for the development of therapeutics. Here, we demonstrate that genes encoding the influenza surface antigens hemagglutinin and neuraminidase have varying stability when cloned into a common RG plasmid and transformed into *Escherichia coli*. Using GFP as a reporter, we demonstrate that *E. coli* expresses the target genes in the RG plasmid at low levels. Incorporating *lac* operators or a transcriptional terminator into the plasmid reduced expression and stabilized the viral genes to varying degrees. Sandwiching the viral gene between two *lac* operators provided the largest contribution to stability and we confirmed the stabilization is Lac repressor-dependent and crucial for subsequent plasmid propagations in *E. coli*. Viruses rescued from the *lac* operator-stabilized plasmid displayed similar kinetics and titers to the original plasmid in two different viral backbones. Together, these results indicate that silencing transcription from the plasmid in *E. coli* helps to maintain the correct influenza gene sequence and that the *lac* operator addition does not impair virus production. It is envisaged that sandwiching DNA segments between *lac* operators can be used for reducing DNA segment instability in any plasmid that is propagated in *E. coli* which express the Lac repressor.

KEYWORDS: *neuraminidase, NA, hemagglutinin, HA, lac operon, cloning toxic genes, influenza virus rescue, influenza A virus, IAVs, regulatory elements, iVEC*

INTRODUCTION

Plasmid-based reverse genetics (RG) systems enable viral genomes to be rapidly modified in a directed manner, providing molecular details about viruses that were previously very difficult to ascertain. These systems were first developed in the 1980's−90's for positive and negative stranded RNA viruses[1,2] and are now commonly used to investigate viral pathogenesis, virus−host interactions, and replication.[3−5] Similarly, viral RG systems have been utilized to introduce changes that attenuate a virus for the development of live virus vaccines or for creating more "customized" vaccines that use a virus to produce and elicit a response against an antigen of choice.[4,6] Despite the numerous advances and applications, viral RGs are still dependent on the ability of *Escherichia coli* to propagate plasmids containing viral genes and/or genome segments, which are ultimately used to produce infectious virus following transfection into an appropriate cell line.

Influenza A virus (IAV) RG systems have been instrumental for addressing key questions about the viral life cycle, virus evolution, and for developing new influenza vaccine strategies.[7−10] The first systems involved the transfection of twelve or sixteen plasmids into mammalian cells;[5,11−13] eight human RNA polymerase I (Pol I) promoter-driven plasmids for transcribing the eight negative-sense viral RNA (vRNA) gene segments and eight or four RNA polymerase II (Pol II) promoter-driven plasmids for transcribing either all the viral

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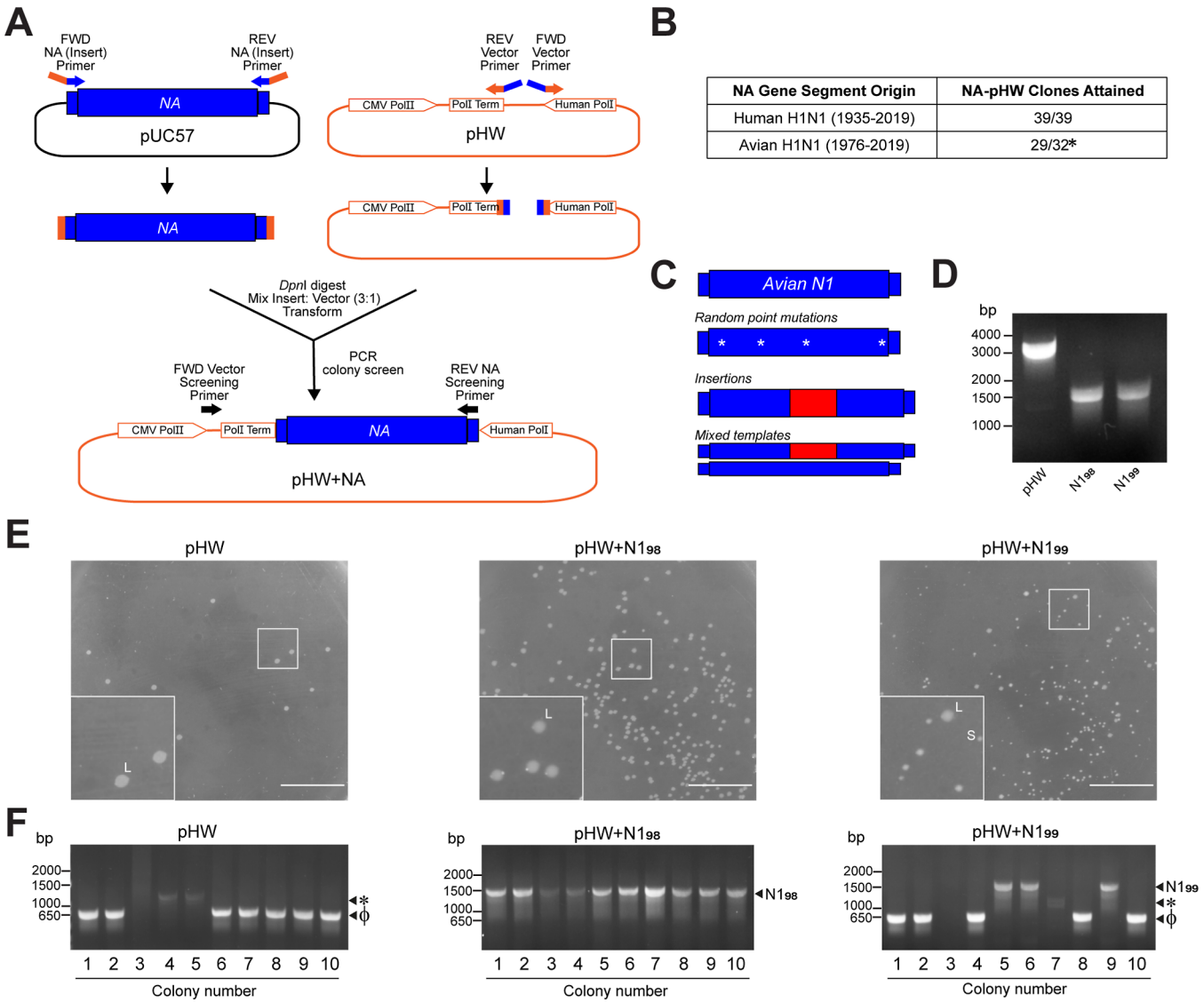

NA Gene Segment Origin	NA-pHW Clones Attained
Human H1N1 (1935-2019)	39/39
Avian H1N1 (1976-2019)	29/32*

Figure 1. Human and avian H1N1 NA plasmid library construction for influenza RG. (A) Schematic of the iVEC cloning strategy for creating the human and avian NA subtype 1 (N1) plasmid libraries. Each N1 gene segment and the pHW plasmid were amplified by PCR prior to *E. coli* transformation. (B) Table displaying the number of human and avian N1 gene segments readily cloned into the pHW plasmid. Asterisk denotes three avian N1 gene segments (from 1983, 1991, and 1999) that required multiple cloning attempts. (C) Diagram showing the typical mutations observed in the 1983, 1991, 1999 avian N1 gene segment clones. (D) Agarose gel (0.8%) image of the PCR-amplified pHW plasmid and $N1_{98}$ and $N1_{99}$ gene segments. (E) Representative images of *E. coli* colonies obtained following transformation with the PCR-amplified pHW plasmid alone and with the indicated N1 gene segments cloned into pHW. Higher magnification insets show the typical large (L) colony size observed for the PHW + $N1_{98}$ transformed bacteria and the atypical smaller (S) colony size of the pHW + $N1_{99}$ transformed bacteria. LB-agar Amp plates images include scale bars that correspond to 1 cm. (F) Agarose gels displaying the NA PCR screening results for 10 randomly selected colonies from each transformation. Bands corresponding to pHW $N1_{98}$ and $N1_{99}$ positive clones are shown (arrowhead) along with products of the wrong size (*) and target-independent PCR products (ϕ) observed from the empty pHW plasmid. NA genes in positive clones were verified by sequencing. Data in panels E and F are representative of three biological repeats.

mRNAs or only the mRNAs encoding for the nucleoprotein (NP) and the three polymerase subunits. These initial plasmids were modified further to create an elegant bidirectional (ambisense) construct that can efficiently generate IAVs from eight plasmids.[14,15] In this system, each pHW plasmid contains one IAV gene segment flanked by a Pol I and a Pol II promoter resulting in the transcription of both vRNAs and mRNAs from all eight gene segments following co-transfection into 293T cells. Although additional plasmid-based RG systems have been developed for IAVs,[16−20] including one that combines synthesized IAV gene segments with backbone plasmids,[21] eight-plasmid systems based on pHW continue to be widely used.

Multiple studies have reported difficulties cloning several IAV gene segments (e.g., PB2, PB1, and HA) into established RG plasmids,[22−24] suggesting particular influenza genes are unstable or "toxic" in *E. coli*. Problems associated with cloning viral genes or cDNAs are not unique to IAVs and have been extensively reported for flaviviruses including Dengue virus, Japanese encephalitis virus, and Kunjin virus.[25,26] However, mechanistic data explaining these observations is lacking and the studies that have investigated unstable viral genes have generally inferred the instability is a result of viral gene

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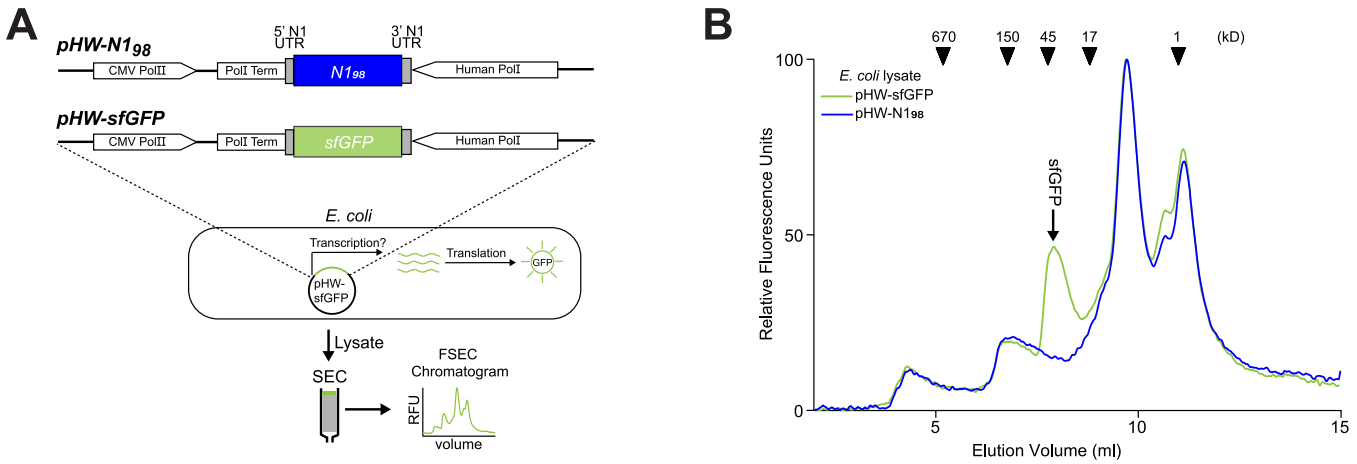

Figure 2. Analysis of gene expression from the pHW plasmid in *E. coli*. (A) Diagram of the assay used for monitoring gene expression from the pHW plasmid in *E. coli*. Bacteria transformed with the pHW plasmid containing $N1_{98}$ or sfGFP genes were grown overnight, lysed and analyzed by FSEC to detect sfGFP expression. (B) Representative FSEC chromatograms of lysates from *E. coli* transformed with the indicated pHW plasmids are displayed. The peak corresponding to sfGFP is indicated. Arrowheads show the elution volumes of the indicated molecular weight standards.

expression in *E. coli*. More recently it has been suggested that transcripts from heterologous genes cloned into plasmids can also contribute to their instability in *E. coli*.[27] Supporting both of these possibilities for IAV genes, cryptic *E. coli* promoter-like sequences have been identified in the Pol II (CMV) promoter,[28,29] which is a common feature in several viral RG plasmids. In addition, regions within multiple viral genomes (e.g., 5′ UTR's of Dengue and Kunjin viruses, the 5′ LTR of Rous sarcoma virus, and the Hepatitis B virus pre-core region) have been shown to facilitate transcription in *E. coli*.[26,30−32]

Several approaches have been reported to increase the stability of IAV RG plasmids in *E. coli*. These include exchanging the *E. coli* origin of replication in the RG plasmid for one that produces low copy numbers,[22,24] using recombination deficient *E. coli* strains for propagating the plasmid,[24] and growing the transformed *E. coli* at low (25−32 °C) temperatures.[23] Although each of these approaches have advantages, none of them provide a universal solution for cloning potentially unstable genes that require amplification in *E. coli* for DNA isolation or protein production.

Our laboratory has frequently encountered difficulties in cloning specific neuraminidase (NA) and hemagglutinin (HA) gene segments from IAVs into a common RG plasmid (pHW). Based on this observation, we examined if the IAV gene segment or features in the RG plasmid are responsible for the instability in *E. coli*. Our results show that genes cloned into the RG plasmid can be transcribed and translated in *E. coli* and that the addition of bacterial regulatory elements can decrease gene transcription and consequently stabilize the problematic NA and HA gene segments in the plasmid. The largest contribution to stability was achieved by sandwiching the influenza gene segment between two *lac* operators in the plasmid and combining it with *E. coli* such as XL10-Gold cells that express an active Lac repressor, which can bind the two *lac* operators cooperatively.[33,34] The modified plasmid efficiently produced IAVs following transfection into mammalian cells, indicating that the Pol I and II promoters were not impaired. Introducing the *E. coli rrnB* terminator sequence[35,36] upstream of the viral gene further increased its stability; however, this also significantly reduced gene expression in mammalian cells that could potentially impact viral production. The broader applications of these plasmid modifications are discussed.

RESULTS

Construction of Human and Avian H1N1 NA Plasmid Libraries. To assess temporal and species related changes in the properties of NA from IAVs, we generated a RG plasmid library carrying NA genes from human and avian H1N1 virus isolates spanning several decades. The library was created by an *in vivo E. coli* cloning (iVEC) approach where the NA subtype 1 (N1) genes were inserted between the human Pol I and cytomegalovirus Pol II promoters of the common influenza RG plasmid pHW[15] (Figure 1A). During construction of the library, three of the avian N1s (1983, 1991, and 1999) proved difficult to clone into the pHW plasmid (Figure 1B). After more extensive screening, putative clones were obtained for these NAs. However, the isolated plasmids typically displayed point mutations, insertions, or heterogeneity in different regions of the NA genes (Figures 1C and S1) indicating that the genes may be unstable in the *E. coli* strain (XL10-Gold) which was used to produce the library.

We examined the cloning problem more thoroughly by comparing the potentially unstable avian N1 gene from 1999 ($N1_{99}$) to a more easily cloned avian N1 gene from 1998 ($N1_{98}$). Although no difficulties were observed in amplifying each NA gene segment or the pHW plasmid (Figure 1D), transformation with a mixture containing the amplified $N1_{99}$ gene and pHW vector (pHW + $N1_{99}$) yielded atypical *E. coli* colonies on LB-agar Amp plates that were much smaller than those that were observed following transformation with a pHW + $N1_{98}$ mixture (Figure 1E). PCR screening of randomly selected large and small colonies from the plates showed that all colonies transformed with pHW + $N1_{98}$ produced a band corresponding with the expected full-length NA gene insert (Figure 1F, middle panel), whereas only a minority (30%) of the colonies transformed with pHW + $N1_{99}$ yielded the expected ∼1500 bp band (Figure 1F, right panel). Subsequent sequencing of the plasmids revealed that the few potential full-length pHW-$N1_{99}$ clones frequently contained point mutations, rendering them unsuitable for viral generation.

Analysis of Gene Expression from the Influenza RG Plasmid in *E. coli*. Previous studies have shown that the CMV promoter in eukaryotic expression plasmids contains *E. coli* promoter-like sequences.[28,29] Therefore, we hypothesized that *E. coli* promoter-like sequences in the Pol II (CMV) promoter

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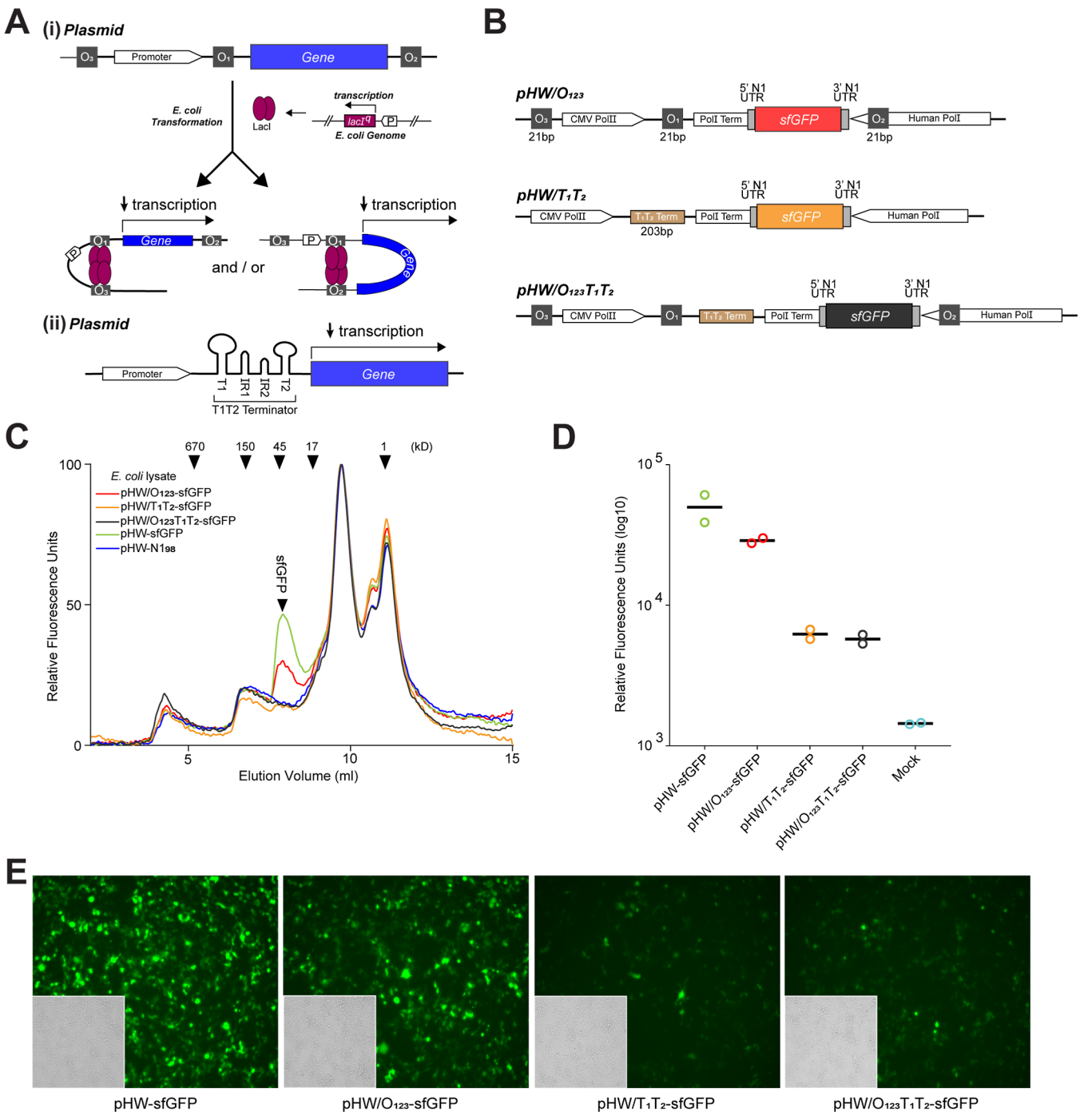

Figure 3. Analysis of gene expression from the pHW plasmid variants in prokaryotic and eukaryotic cells. (A) Diagrams showing how plasmid-derived gene transcription can be minimized by positioning (i) three cooperative wild type *lac* operator sequences or (ii) the *E. coli rrnB* transcriptional terminator around a gene of interest. Note there are more possible LacI interactions with the *lac* operator than shown. (B) Schematics of pHW plasmid variants carrying sfGFP with three *lac* operator sequences (pHW/O_{123}), the *rrnB* transcriptional terminator (pHW/T_1T_2), and the *lac* operators combined with the terminator (pHW/$O_{123}T_1T_2$). (C) FSEC chromatograms of *E. coli* lysates carrying the indicated sfGFP pHW plasmid variants. The peak corresponding to sfGFP is indicated. FSEC data are representative of two biological and three technical repeats. (D) GFP fluorescence of 293-T cell lysates prepared 60 h post-transfection with the indicated sfGFP pHW plasmid variants. Data are from two biological repeats. (E) Representative images showing GFP fluorescence in 293-T cells 60 h post-transfection with the indicated sfGFP pHW plasmid variants. Insets contain brightfield images of the confluent cell layer.

of the pHW plasmid may lead to the expression and subsequent instability of influenza genes in *E. coli*. To test our hypothesis, we transformed *E. coli* with a pHW reporter plasmid (pHW-sfGFP) that encodes the robust super folder green fluorescent protein (sfGFP)[37−39] and a control plasmid (pHW-$N1_{98}$) harboring a stable NA gene (Figure 2A). No sfGFP fluorescence was detected in the *E. coli* using either a plate reader or by imaging the colonies on a plate, indicating any potential sfGFP expression was low and masked by the inherent fluorescence of the *E. coli*.[40,41] To address this possibility we used fluorescent size exclusion chromatography (FSEC)[42] to monitor the sfGFP expression in whole cell

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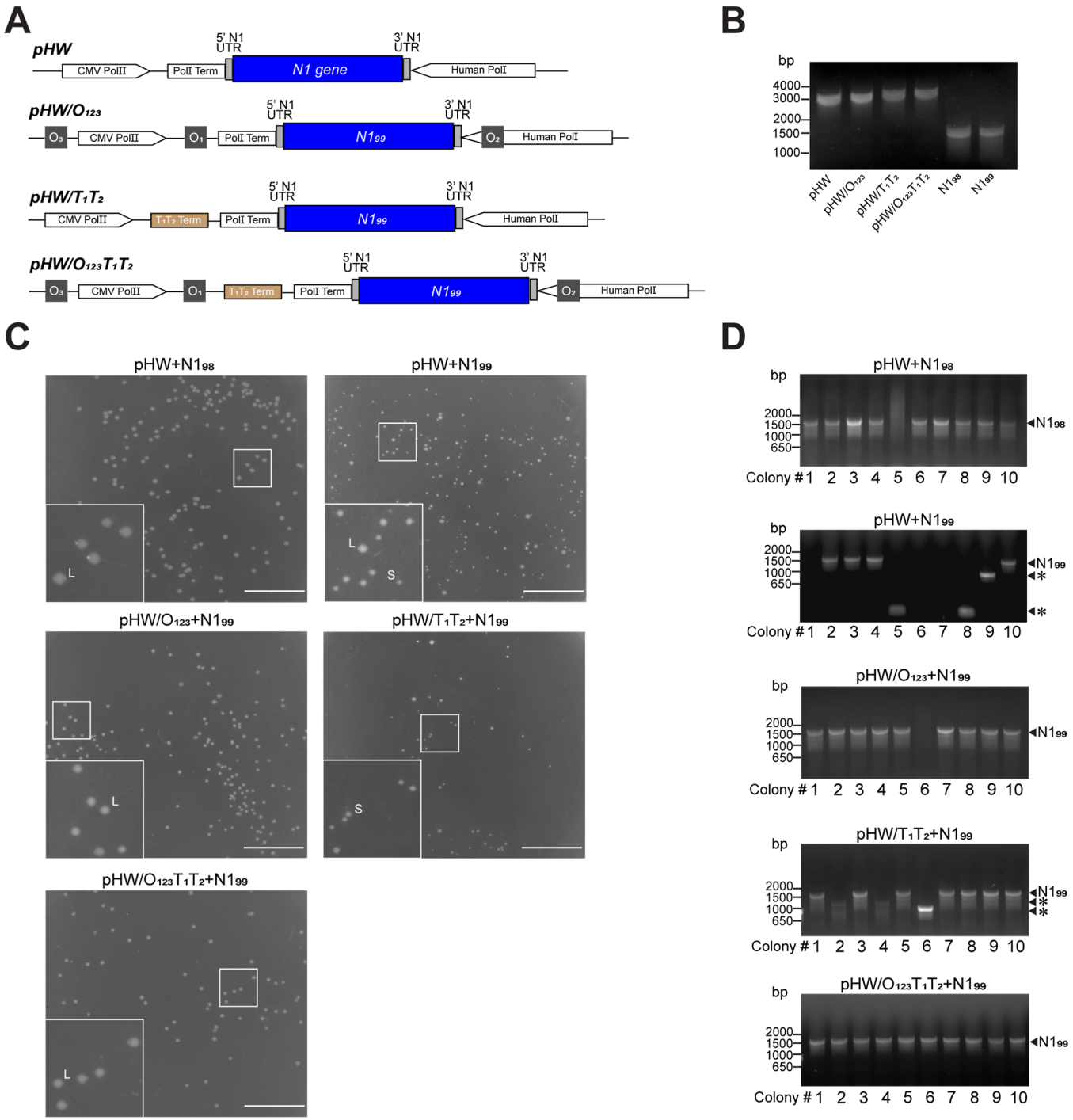

Figure 4. pHW plasmid variants differ in the ability to accommodate the $N1_{99}$ gene segment. (A) Diagram of the N1 pHW plasmid variants that were generated by iVEC. UTRs are from the N1 gene segment. (B) Agarose gel (0.8%) of the PCR-amplified pHW plasmid variants and indicated N1 gene segments. (C) Representative images of *E. coli* colonies that were obtained following transformation with the indicated pHW plasmid variants. Higher magnification insets show the typical large (L) colony size and the atypical small (S) colony sizes that were observed on the LB-agar Amp plates. Scale bars correspond to 1 cm. (D) Agarose gels displaying the PCR screening results of 10 randomly selected colonies from each transformation. Bands corresponding to the predicted NA gene size are indicated. Asterisks denote bands that are not of the expected size. NA genes in positive clones were verified by sequencing. Data in panels C and D are representative of three biological repeats.

bacterial lysates (Figure 2A). Following the FSEC analysis, a clear peak corresponding to sfGFP was only observed from the bacteria transformed with pHW-sfGFP (Figure 2B), indicating that genes inserted into the pHW plasmid are expressed in *E. coli* at a low but detectable level.

Based on the sfGFP results, we attempted to silence gene expression from the pHW plasmid in *E. coli* by introducing the three natural *lac* operator sequences[43] and/or the widely used transcriptional terminator from the *rrnB* gene[35,36] into the pHW plasmid backbone (Figures 3A and S2). In one of the modified plasmids, a single *lac* operator sequence was positioned upstream of the Pol II promoter, another was positioned before the sfGFP gene, and a third was positioned downstream of the sfGFP gene (Figure 3B, pHW/O_{123}). This

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configuration was chosen because it mimics the natural *lac* operon and allows for the binding of multiple Lac repressors which can interact with each other to form loops in the DNA and potentially enhance transcriptional repression.[33,34,44] The second modified plasmid contained the transcription termination region of the *E. coli rrnB* gene[35,36] upstream of the sfGFP gene (Figure 3B, pHW/T_1T_2), and the third modified plasmid contained the three *lac* operators and the terminator (Figure 3B, pHW/$O_{123}T_1T_2$). To analyze how the different modifications affected expression, the plasmids were transformed into *E. coli* that express the Lac repressor and the sfGFP level in each whole cell lysate was measured by FSEC. Compared to bacteria transformed with the non-modified plasmid pHW-sfGFP, the GFP signal was reduced by ∼50% in the bacteria transformed with pHW/O_{123}-sfGFP and by ∼95% in the bacteria transformed with pHW/T_1T_2-sfGFP (Figure 3C). The GFP signal was further reduced in the bacteria transformed with pHW/$O_{123}T_1T_2$-sfGFP (Figure 3C), indicating the operator and terminator combination almost completely silences transcription from the pHW plasmid in *E. coli*.

To determine if the presence of the *lac* operators or the transcriptional terminators hindered the gene expression driven by the Pol II promoter, we transfected 293T cells with each of the plasmids. The transfected cell lysate fluorescence (Figure 3D) and live cell imaging (Figure 3E) both showed that the GFP signal with pHW/O_{123}-sfGFP transfected cells was ∼60% of the pHW-sfGFP transfected cells. In the cells transfected with either pHW/T_1T_2-sfGFP or pHW/$O_{123}T_1T_2$-sfGFP, the GFP signal was reduced to ∼10% of the pHW-sfGFP transfected cells, indicating that the *lac* operators have less of a negative impact on mRNA transcription from the plasmid in eukaryotic cells.

Stability of the Avian $N1_{99}$ Gene Segment in the Modified pHW Plasmids. To test if the regulatory elements improved the ability to clone the problematic avian $N1_{99}$ gene, we compared the cloning results using the pHW plasmid with the three modified pHW plasmids (Figure 4A). Despite the presence of highly structured sequences,[36] no difficulties were observed in amplifying the three modified pHW plasmids by PCR (Figure 4B). Transformations with the pHW/O_{123} + $N1_{99}$ and pHW/$O_{123}T_1T_2$ + $N1_{99}$ mixtures both produced *E. coli* colonies that were larger than the pHW + $N1_{99}$ colonies and more similar in size to the colonies obtained from the pHW + $N1_{98}$ control transformation (Figure 4C). Colonies from the *E. coli* transformed with the pHW/T_1T_2 + $N1_{99}$ mixture remained small (Figure 4C), which was unexpected based on the sfGFP results. The phenotypic observations were supported by a PCR screen of randomly selected large and small colonies as almost 100% of the pHW/O_{123} + $N1_{99}$ and pHW/$O_{123}T_1T_2$ + $N1_{99}$ colonies yielded a band corresponding to the full-length $N1_{99}$ insert, whereas only 65% of the pHW/T_1T_2 + $N1_{99}$ colonies and 52% of pHW + $N1_{99}$ colonies yielded the expected band (Table 1 and Figure 4D). The high positive screen rates for pHW/O_{123} + $N1_{99}$ and pHW/$O_{123}T_1T_2$ + $N1_{99}$ were in line with the 94% positivity rate for the control transformation with pHW + $N1_{98}$ (Table 1), suggesting the *lac* operators effectively minimize the instability in *E. coli* that is caused by the transcription of the NA gene from the pHW plasmid.

Stability of HA Gene Segments in pHW/O_{123}. Similar to a previous study,[24] we also experienced problems cloning two different HA (H1 and H6) gene segments into the pHW plasmid (Figure S3), making them ideal targets for testing the general ability of pHW/O_{123} to increase the stability of influenza genes (Figure 5A). As expected, no difficulties were observed in amplifying pHW/O_{123} or the H1 and H6 gene segments (Figure 5B). Following transformation with pHW/O_{123} + H1, the colonies were noticeably larger than those transformed with pHW + H1 (Figure 5C, top two panels). For the H6 gene, the results were somewhat different, but still consistent. Transformation with pHW/O_{123} + H6 yielded numerous small colonies that were found to contain H6, whereas the transformation with pHW + H6 produced very few large colonies and almost no small colonies (Figure 5C, bottom two panels). Random PCR colony screening showed that 100% of the colonies transformed with pHW/O_{123} + H1 and 87% of the colonies transformed with pHW/O_{123} + H6 produced products of the expected length, compared to 70% for pHW + H1 colonies and 15% for pHW + H6 colonies (Table 2 and Figure 5D). These screening results combined with the phenotypic observations confirmed that the *lac* operators in pHW can also stabilize HA genes, likely by silencing expression of the HA gene from the plasmid in *E. coli*.

Table 1. Colony Screening of NA Gene Segments Cloned into the pHW Plasmid Variants[a]

Plasmid	Positive full-length clones
pHW + $N1_{98}$	31/33 (93.9%)
pHW + $N1_{99}$	17/33 (51.5%)
pHW/O_{123} + $N1_{99}$	21/23 (91.3%)
pHW/T1T2 + $N1_{99}$	15/23 (65.2%)
pHW/O_{123}T1T2 + $N1_{99}$	23/23 (100%)

[a]NA gene segments and the indicated pHW variant were amplified by PCR, mixed, and transformed into *E. coli*. Putative positive full-length clones were determined by a PCR screen of randomly selected colonies using a primer pair that targets an upstream region in pHW (pHW Screen) and the 3′ end of the NA insert (NA reverse).

Dependence of H6 Gene Segment Stability on *lac* Operator Locations in pHW/O_{123}. To investigate if all three *lac* operators are essential for H6 gene segment stability, we created three additional variants of pHW/O_{123} (Figure 6A). One contained two operators located on either side of the gene segment (pHW/O_{12}), the second contained two operators on either side of the Pol II promoter sequence (pHW/O_{13}) and the third was a control that contained only one operator upstream of the Pol II promoter sequence (pHW/O_3). Transformation with pHW/O_{13} + H6 or pHW/O_3 + H6 both yielded few large colonies similar to pHW + H6. In contrast, transformation with pHW/O_{12} + H6 or pHW/O_{123} + H6 both produced numerous small colonies, suggesting that the operators on both sides of the H6 gene segment are crucial for its stability (Figure 6B). As expected, the control transformation with pHW + $N1_{98}$ yielded mostly large bacterial colonies. We then pooled five large and five small colonies from each plate and screened for the presence of the full-length H6 insert by PCR (Figure 6C). In all instances except one (pHW/O_3 + H6), the pooled small colonies produced a band at the expected size for full-length H6, whereas all the pooled large colony preparations yielded a much smaller band. Taken together, these data suggest that cryptic promoter-like sequences within the H6 gene segment facilitate its expression in *E. coli* and that the expression is likely toxic, resulting in the formation of small *E. coli* colonies even when transcription of the H6 gene segment is reduced. Supporting this hypothesis,

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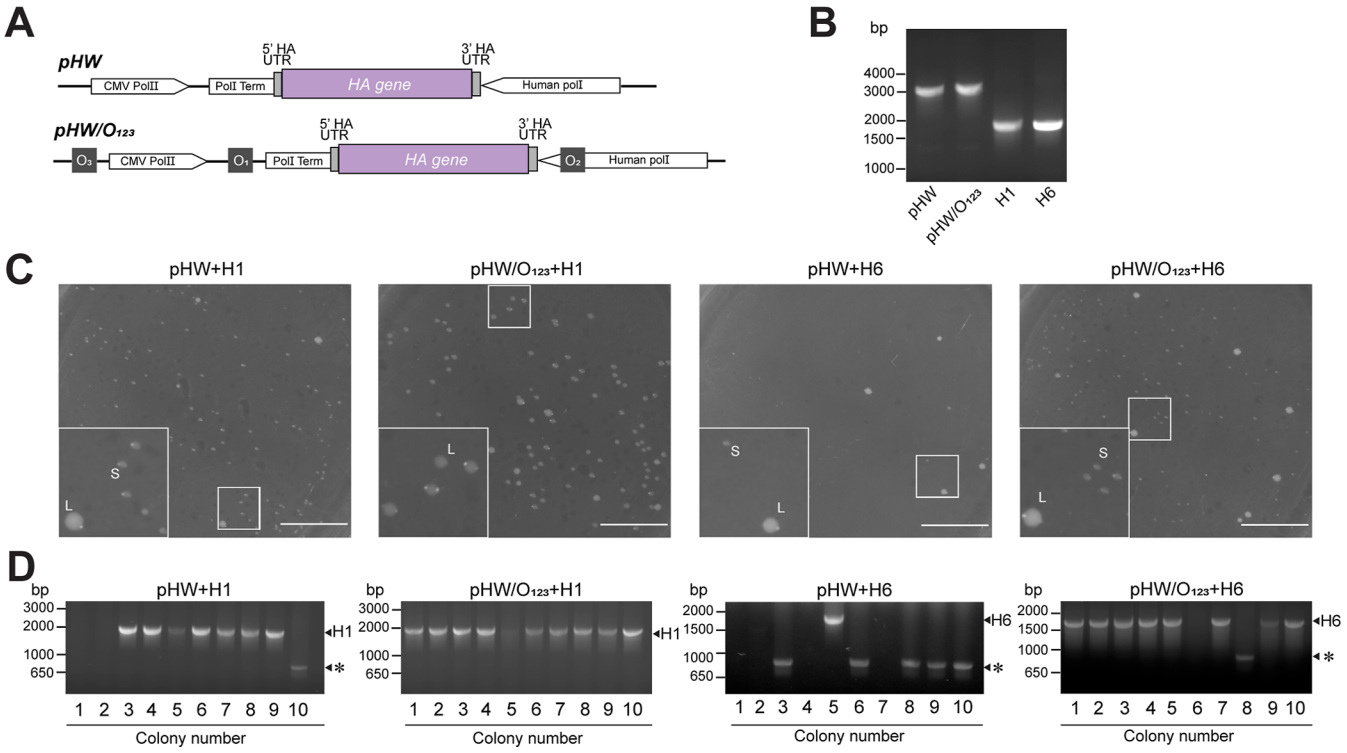

Figure 5. HA (H1 and H6) gene segments are more readily introduced into the pHW/O_{123} plasmid variant. (A) Diagram of the HA (H1 and H6) pHW and pHW/O_{123} plasmid variants that were generated by iVEC. 5′ and 3′ HA gene segment UTRs are indicated. (B) Agarose gel (0.8%) image of the indicated PCR-amplified pHW plasmids and HA gene segments. (C) Representative images of *E. coli* colonies obtained following transformation with the indicated pHW plasmid variants. Higher magnification insets show the large (L) and atypical small (S) colony sizes that were observed on the LB-agar Amp plates following transformation. Scale bars correspond to 1 cm. (D) Agarose gel (0.8%) images displaying the PCR screening results of 10 randomly selected colonies from each transformation. Bands corresponding to the HA gene predicted size are indicated. Asterisks denote bands of the incorrect size. HA genes in positive clones were verified by sequencing. Data in panels C and D are representative of three biological repeats.

Table 2. Colony Screening of HA Gene Segments Cloned into the pHW and pHW/O_{123} Plasmids[a]

Plasmid	Positive full-length clones
pHW + H1	7/10 (70.0%)
pHW/O_{123} + H1	10/10 (100.0%)
pHW + H6	2/13 (15.4%)
pHW/O_{123} + H6	13/15 (86.7%)

[a]HA gene segments and the indicated pHW variant were amplified by PCR, mixed, and transformed into *E. coli*. Putative positive full-length clones were determined by a PCR screen of randomly selected colonies using a primer pair that targets an upstream region in pHW (pHW screen) and the 3′ end of the HA insert (HA reverse).

low level transcription has been reported from most of the *E. coli* genome, indicating that promoter recognition by the *E. coli* RNA polymerase is rather promiscuous.[45]

All of our data thus far suggests that expression from the influenza genes in the pHW plasmid is responsible for the observed instability and cloning difficulties. To test this more directly, we equally divided the pHW/O_{123} + H6 transformed bacteria between plates that lacked and contained isopropyl β-D-1-thiogalactopyranoside (IPTG), which prevents binding of the Lac repressor to the *lac* operator[46] (Figure 6D). In line with our prior results (Figure 6B), *E. coli* transformed with pHW/O_{123} + H6 and grown on agar plates lacking IPTG yielded numerous small colonies. However, only a few large colonies were observed when the same *E. coli* were grown on LB-agar Amp plates containing IPTG, similar to the transformation with pHW + H6. Plates (+/− IPTG) containing pHW + $N1_{98}$ transformed *E. coli* were included as a control and no differences in colony morphology were observed, indicating the stability of the H6 gene segment in pHW/O_{123} is dependent on the binding of the Lac repressor to the *lac* operators.

Influenza Virus Rescue Using pHW/O_{123}. Addition of two or more *lac* operators in the pHW plasmid made the largest contribution to stability (Figures 4D and 5D) and showed the least impact on expression in mammalian cells (Figure 3E). Therefore, we compared the viral rescue kinetics from the pHW/O_{123} plasmid to the parental pHW plasmid. For the initial analysis, viruses were generated using either the pHW/O_{123}-$N1_{99}$ or pHW/O_{123}-H6 plasmids together with seven pHW backbone plasmids that encode for gene segments from the H1N1 IAV strain A/WSN/1933 (WSN). Both viruses generated with a pHW/O_{123} plasmid ($WSN^{N1/99}$* and $WSN^{H6\ N1/18}$*) showed a slight delay in the production of NA activity and HAU titers (Figure 7A). However, at later time points, NA activity and HAU titers equaled or exceeded those for the viruses ($WSN^{N1/99}$ and $WSN^{H6\ N1/18}$) generated exclusively with pHW plasmids, indicating that viruses rescued from the pHW/O_{123} plasmid reach similar titers to pHW rescued viruses despite the slightly slower kinetics. We then sent the same pHW-H6 and pHW/O_{123}-H6 plasmids for large scale DNA production to determine if the plasmids could be propagated in an independent lab setting. Virus ($WSN^{H6\ N1/18\#}$) was successfully rescued from the commercial

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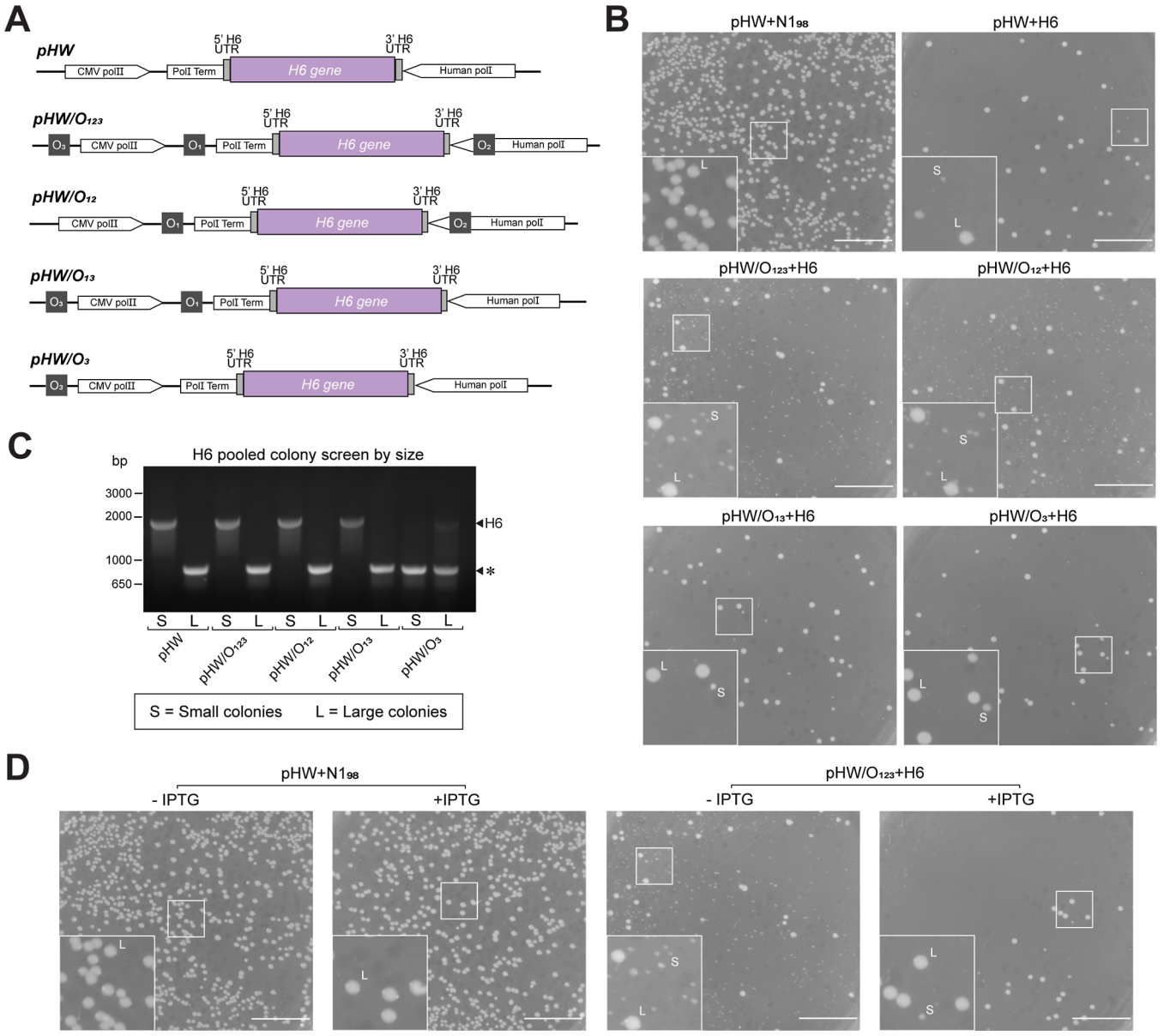

Figure 6. Location and number of *lac* operators are crucial for H6 insertion into the modified pHW plasmid. (A) Diagram of the H6 pHW plasmid variants that were generated with different combinations of the three *lac* operators by iVEC. 5′ and 3′ HA gene segment UTRs are indicated. (B) Representative images of *E. coli* colonies obtained following transformation with the indicated pHW plasmid variants. Higher magnification insets show the large (L) and small (S) colony sizes observed on the LB-agar Amp plates. Scale bars correspond to 1 cm. (C) Agarose gel (0.8%) displaying the PCR screening results of five pooled L and S colonies from each transformation. Bands corresponding to the H6 gene predicted size are indicated. The asterisk denotes a band of an unexpected size. (D) Representative images of *E. coli* colonies obtained from transformation with the indicated plasmids and overnight growth on LB-agar Amp plates with and without IPTG. Higher magnification insets show the L and S colonies that were observed. Scale bars correspond to 1 cm. Data in panels C and D are representative of three biological repeats.

pHW/O_{123}-H6 plasmid DNA, which contained the correct H6 sequence (Figure 7A). In contrast, the commercial pHW-H6 plasmid DNA contained an insertion in the H6 gene making it unsuitable for virus rescue, further confirming that influenza genes are more stable in the pHW/O_{123} plasmid.

All rescued viruses were passaged in embryonated eggs to determine if any differences were observed in viral propagation or protein content. Each virus rescued from the pHW/O_{123} plasmid preparations ($WSN^{N1/99}$*, $WSN^{H6\ N1/18}$*, and $WSN^{H6\ N1/18\#}$) produced NA activities, HAU, and infectious titers that were equivalent or higher than the analogous viruses ($WSN^{N1/99}$ and $WSN^{H6\ N1/18}$) produced entirely from pHW plasmids (Figure 7B and Table 3). SDS-PAGE analysis of the isolated viruses showed that the viral protein content was similar and that the H6 and $N1_{99}$ proteins resolved at the expected molecular weights (Figure 7C), indicating pHW/O_{123} retains the ability to produce recombinant virus.

To examine if the delay in the viral rescue kinetics would be exacerbated in other settings, we compared the rescue of WSN from eight pHW/O_{123} plasmids versus the eight parental pHW plasmids. In addition, we compared the viral rescue from the pHW/O_{123}-$N1_{99}$ plasmid versus the pHW-$N1_{99}$ plasmid in combination with seven different pHW backbone plasmids from the H1N1 IAV strain A/PR/8/1934 (PR8). During the rescue, the WSN viruses generated by the eight pHW/O_{123} plasmids and the eight pHW plasmids both displayed similar

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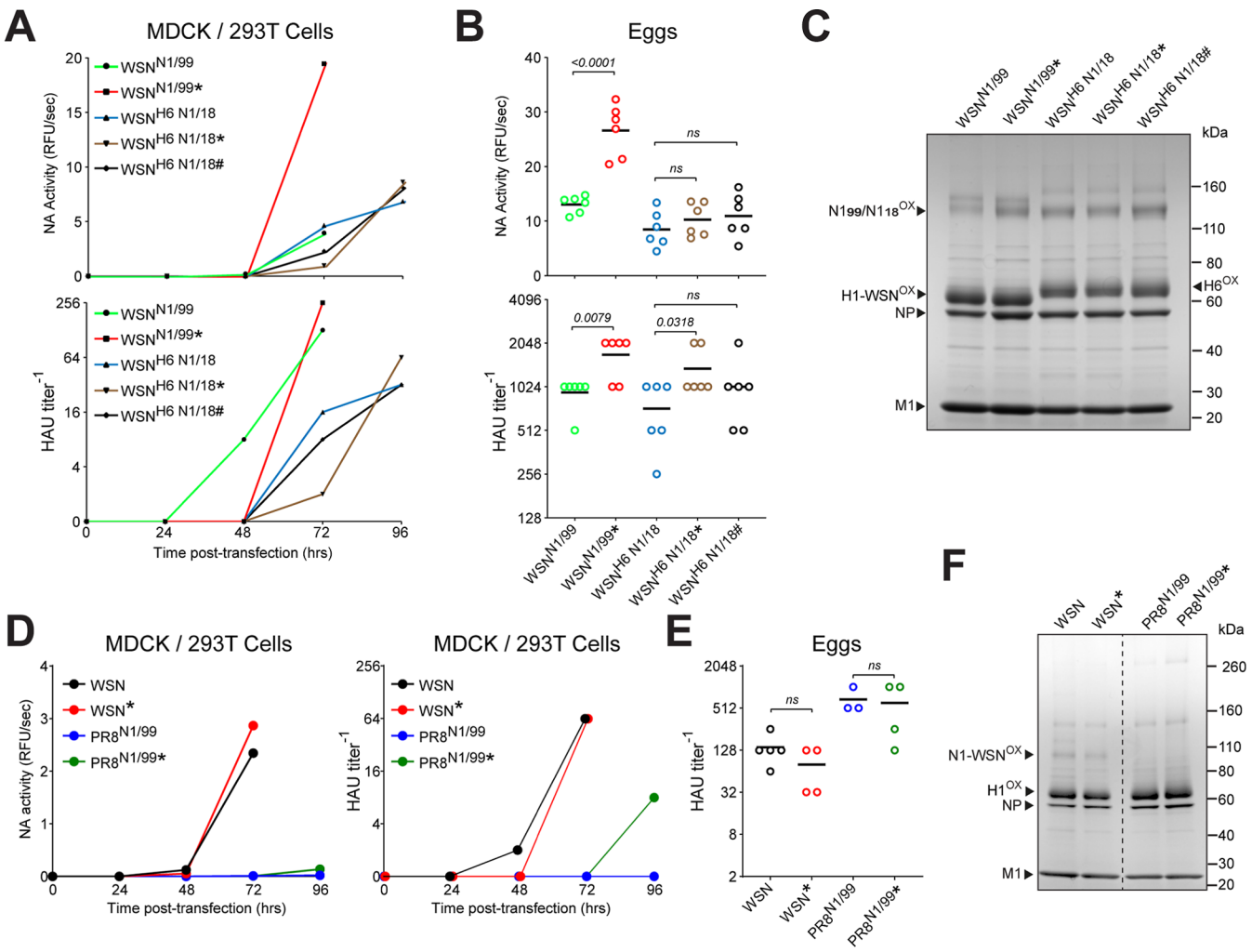

Figure 7. Influenza virus rescue using pHW/O_{123}. (A) NA activities and HAU titers of the indicated viruses during the RG rescue are displayed. Measurements were from equal cell culture supernatant volumes at the indicated times. Asterisks indicate viruses ($WSN^{N1/99}$* and $WSN^{H6\ N1/18}$*) generated with the pHW/O_{123}-$N1_{99}$ and pHW/O_{123}-H6 plasmids, respectively. Hashtag (#) represents a virus ($WSN^{H6\ N1/18\#}$) generated with a commercial pHW/O_{123}-H6 plasmid preparation. (B) Indicated viruses were passaged in eggs for 72 h and the NA activities and HAU titers were measured using equal allantoic fluid volumes. Individual egg data is displayed with the mean (bar). (C) Image of a non-reduced Coomassie-stained SDS-PAGE gel (4−12%) containing the indicated virions (~5 μg) isolated by sedimentation. Oxidized (OX) forms of the NA and HA proteins are indicated along with the viral proteins NP and M1. (D) NA activities and HAU titers of the indicated viruses during RG rescue are displayed. Measurements were from equal cell culture supernatant volumes. Asterisks denote viruses generated with eight pHW/O_{123} plasmids (WSN*) or the pHW/O_{123}-$N1_{99}$ plasmid combined with seven PR8 pHW plasmids ($PR8^{N1-99}$*). (E) Viruses were passaged in eggs for 72 h, and the HAU titers were measured from equal allantoic fluid volumes. Data from uninfected eggs were excluded. Each bar corresponds to the mean. (F) Non-reduced Coomassie-stained SDS-PAGE gel image of the indicated virions (~5 μg) isolated by sedimentation. All *P* values were calculated from a two-tailed unpaired *t*-test (95% CI).

NA activities and HAU titers, indicating that the delay in the rescue kinetics is not amplified when pHW/O_{123} is used as an eight plasmid system (Figure 7D). In contrast, only the PR8 virus generated from the pHW/O_{123}-$N1_{99}$ plasmid ($PR8^{N1/99}$*) produced measurable NA activity and HAU titers by 96 h post-transfection (Figure 7D). Upon passaging the cell culture media from the rescues in eggs, all four viruses grew, and no significant differences were observed in the HAU titers of the infected eggs (Figure 7E) or the protein profiles of the isolated virions (Figure 7F). Together, these results show that the more stable pHW/O_{123} plasmid can be used in combination with the parental pHW RG system or as a stand-alone eight plasmid system to generate recombinant viruses without any substantial loss in efficiency.

pHW/O_{123} Allows Unstable Influenza Genes to be Propagated in *E. coli*. During this study, small scale preparations of pHW-H6 and pHW/O_{123}-H6 were sent for commercial DNA production; however substantial changes were found in the pHW-H6 plasmid DNA we received. Based on this observation we re-transformed *E. coli* with the sequence and PCR-verified (Figure 8A) small scale preparations of pHW-H6 and pHW/O_{123}-H6 to determine the stability of the H6 gene segment during propagation of the plasmid DNA in bacteria. Almost all colonies (99.5%) transformed with pHW/O_{123}-H6 showed the expected small phenotype (Figure 8B), whereas almost all colonies (96.5%) transformed with pHW-H6 were large (Figure 8B). PCR screening of randomly selected small and large *E. coli* colonies transformed with pHWO_{123}-H6 produced a band at the expected size for full-length H6, whereas all colonies transformed with pHW-H6 yielded a larger than expected band, suggesting that the increased stability provided by pHW/O_{123} is equally crucial for

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Table 3. Infectious Titers of Rescued Viruses Following a Single Passage in Eggs[a]

Rescued virus	$TCID_{50}$/mL	EID_{50}/mL
$WSN^{N1/99}$	1.4×10^6	n.d.
$WSN^{N1/99*}$	2.3×10^7	n.d.
$WSN^{H6\ N1/18}$	3.3×10^4	6.8×10^7
$WSN^{H6\ N1/18*}$	5.3×10^4	6.8×10^7
$WSN^{H6\ N1/18\#}$	2.5×10^4	n.d.

[a]Median tissue culture infectious doses per milliliter ($TCID_{50}$/mL) were determined using MDCK cells in 96-well plates, and the results represent the mean of two independent analysis. Median egg infectious doses per milliliter were determined using specific pathogen-free eggs. Asterisks indicate viruses rescued from the pHW/O_{123}-N1/99 and pHW/O_{123}-H6 plasmids, respectively. The hashtag (#) represents the virus rescued from a commercial preparation of pHW/O_{123}-H6. n.d.—not determined.

plasmid propagation (Figure 8C). Taken together, these data demonstrate that the pHW/O_{123} plasmid can readily accommodate unstable influenza genes while maintaining the ability to efficiently generate recombinant IAVs.

DISCUSSION

All influenza RG systems are dependent on *E. coli* for generating and propagating the plasmids that contain the viral gene segments. Our data demonstrates that the stability of NA and HA genes vary during plasmid propagation in *E. coli*, resulting in unwanted point mutations, deletions, or insertions. Similar issues have been reported for other IAV genes,[22−24] suggesting that influenza RG plasmids may frequently be subject to selection pressure in *E. coli* and this could contribute to unexpected and unwanted substitutions in the viruses that are generated by transfecting these plasmids into mammalian cells. Supporting this premise, we showed that *E. coli* express genes in the RG plasmid and that the addition of transcriptional regulatory elements can mitigate expression and influenza gene instability in *E. coli*. The largest contribution to gene stability in *E. coli* with the least impact on expression in mammalian cells was achieved by sandwiching the influenza gene between *lac* operators, and this modification was crucial for subsequent plasmid propagation in *E. coli*. We confirmed that the stabilization requires the Lac repressor and that viruses rescued from the modified plasmid (pHW/O_{123}) with a third *lac* operator upstream of the Pol II promoter can reach high titers following a single passage in eggs. These findings indicate that the *lac* operators can stabilize the influenza genes in *E. coli* that express the Lac repressor and that the approach of stabilizing heterologous DNA sequences by sandwiching them between *lac* operators can be used in any plasmid.

Numerous viral and non-viral genomic sequences have been reported to be unstable or "toxic" in *E. coli*,[28,47−51] and multiple approaches for cloning unstable genes have been developed [reviewed in ref 52]. These include in vitro synthesis of the gene,[21] modifying regions of the toxic gene[47−49] and/or the use of recombination deficient *E. coli* strains,[24,53] low copy number plasmids,[24,54,55] plasmids containing negative selection markers[24] or terminators,[50] lower *E. coli* culturing temperatures,[23] and more aggressive antibiotic selection regimens.[22] For stabilizing influenza genes, most studies have used low copy number plasmids,[22,24] recombination deficient *E. coli* strains,[24] or reduced the *E. coli* culturing temperature.[23] However, none of these approaches have been shown to prevent *E. coli* from expressing the viral genes, increasing the likelihood of the bacteria modifying the RG plasmids to mitigate "toxicity" during plasmid propagation. Along these lines, the pHW plasmid contains an *E. coli* origin of replication (OriC) from pBR322 that produces low to medium copy numbers,[56] and yet the instability of the NA and HA genes were exacerbated upon propagation in *E. coli* and retransformation. Despite this observation, these strategies are not mutually exclusive, making it possible that we could further increase gene stability in our plasmid by introducing a low copy number OriC or lowering the *E. coli* incubation temperature.

The native *lac* operon in the *E. coli* genome achieves full repression by using three operators that function cooperatively.[33,34] Therefore, we expected that the addition of three operators would provide higher gene stability in the RG plasmid than the common approach of using one *lac* operator. However, we did not expect that the terminator would only show some benefit in the presence of the three operators as we found the terminator reduced the GFP reporter expression in

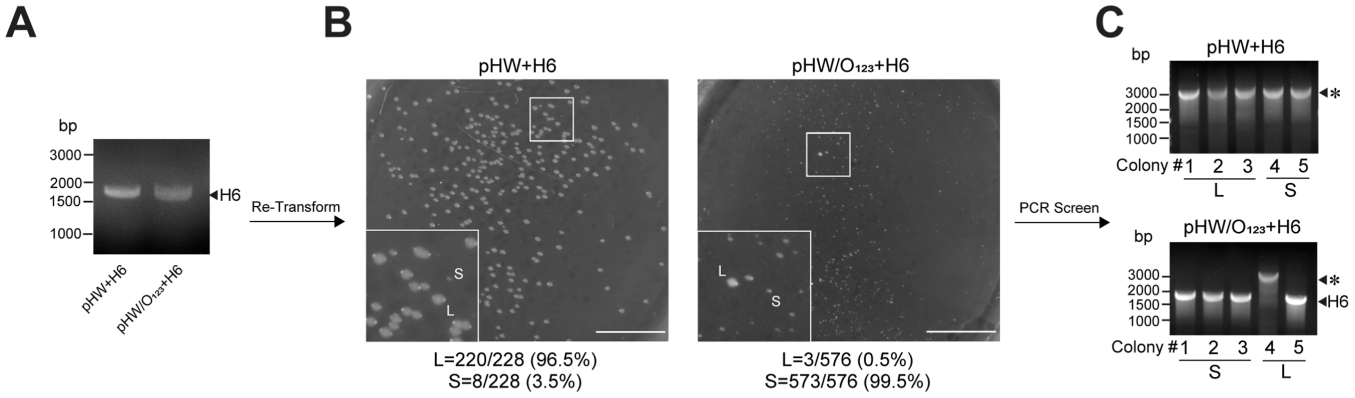

Figure 8. H6 gene segment stability in pHW and pHW/O_{123} following re-transformation. (A) Agarose gel (0.8%) image of the PCR-amplified H6 gene segment from the sequence-verified H6 pHW and H6 pHW/O_{123} plasmids that were used for *E. coli* transformation. (B) Representative images of *E. coli* colonies that were obtained on LB-agar Amp plates following transformation with the sequence and PCR verified H6 pHW and H6 pHW/O_{123} plasmids. Insets show higher magnification of the large (L) and small (S) colony sizes that were observed. Scale bars correspond to 1 cm. (C) Agarose gel (0.8%) images of the PCR screening results from five randomly selected L and S colonies from each LB-agar Amp plate. Bands corresponding to the predicted H6 gene size are indicated. Asterisks denote a band that is not of the expected size.

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E. coli by ∼95%, whereas the operators only reduced it by ∼50%. This unexpected result suggests that some unstable influenza genes may contain cryptic bacterial promoters that express transcripts or gene products in *E. coli* that are "toxic". The presence of cryptic transcriptional or translational regulatory elements within a viral genomic segment is not without precedent,[26,30−32] and any of these possibilities could explain why the *lac* operator located after the influenza genes was instrumental for stabilization in the pHW/O_{123} plasmid.

The benefit of the *lac* operators was most clearly shown for the H6 gene which was also used to verify the Lac repressor which is crucial for mitigating the instability. The same construct was used to test if the influenza genes in the pHW/O_{123} plasmid showed increased stability outside of our laboratory. For this test, we sent sequence-verified pHW/O_{123}-H6 and pHW-H6 plasmids to a commercial service for large-scale DNA preparation. However, only the pHW/O_{123}-H6 plasmid was readily produced without mutations, indicating the robustness of the pHW/O_{123} plasmid. Additionally, it should be noted that the commercial service amplified the plasmids in an *E. coli* DH5-alpha strain rather than the XL-10 Gold strain used in our laboratory, confirming that the instability of influenza genes in the original plasmid (pHW) and the stabilizing effect of the modified plasmid (pHW/O_{123}) are not specific to a single *E. coli* cloning strain.

Cooperative repression in the native *lac* operon is thought to occur by the formation of loops in the DNA that are created by interactions between the bound Lac repressor tetramers.[33,34,44] We envisage that this mechanism also contributes to increased gene stability in the pHW/O_{123} plasmid. Additionally, interactions between the bound repressors could also facilitate the aggregation of multiple pHW/O_{123} copies that are present in an *E. coli*,[44] making the genes inaccessible to the transcription machinery. Since both mechanisms are likely to function in the context of any plasmid or DNA sequence, we speculate that the *lac* operator strategy for stabilizing influenza genes in pHW would also function to stabilize any heterologous DNA sequences in other plasmids that are propagated in *E. coli* which express the Lac repressor. While additional modifications such as the inclusion of the Lac repressor gene on the plasmid itself could offer further improvements to the strategy, our results show that minimizing gene expression in *E. coli* provides a clear advantage for plasmid-based influenza RG systems.

METHODS

Reagents. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (P/S), Opti-MEM I (OMEM), Simple Blue Stain, Novex 4−12% Tris-glycine SDS-PAGE gels, Novex Sharp Unstained Protein Standard, GeneRuler 1 kb Plus DNA Ladder, LB Medium Dehydrated Capsules, and the Phusion High-Fidelity DNA Polymerase were all purchased from Thermo Fisher Scientific. His-tagged Pfu X7 DNA Polymerase was prepared in-house by immobilized metal affinity chromatography for routine PCR-based bacterial colony screening. XL10-Gold Ultracompetent cells were acquired from Agilent Technologies, Inc. Note XL10-Gold cells have a mutation in the *lacI* gene promoter ($lacI^q$), resulting in higher Lac repressor levels.[57] SIGMAFAST EDTA-free Protease Inhibitor cocktail tablets and IPTG were obtained from Sigma-Aldrich. *Dpn*I, TransIT-LT1 transfection reagent, and 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) were obtained from New England Biolabs, Mirus Bio, and Cayman Chemicals, respectively. Specific-pathogen-free (SPF) eggs and turkey red blood cells (TRBCs) were purchased from Charles River Labs and the Poultry Diagnostic and Research Center (Athens, GA), respectively. All cloning and screening primers (Table S1) used in this study were synthesized by Integrated DNA Technologies.

Plasmids and Constructs. The eight WSN (A/WSN/33) and PR8 (A/PR/8/34) RG plasmids were provided by Dr. Robert Webster (St. Jude Children's Research Hospital). The RG plasmids were sequenced, and the GenBank Identifications along with the details for generating the NA (N1-BR18; GISAID ID: EPI1212833) RG plasmid were described previously.[58] To create the NA [Human H1N1 (1935−2019), Avian H1N1 (1976−2019)] and HA [H1-BR18 (GISAID ID: EPI1212834) and H6 (GenBank ID: CY087752.1)] RG plasmids, the NA and HA gene segments with their respective 5′ and 3′ untranslated regions (UTR's) were amplified by PCR using gene specific primers with overhangs matching the plasmid insertion sites (Table S1) and commercially synthesized as gene templates in pUC57 (GenScript USA). The amplified genes were then cloned into a PCR-amplified pHW2000 (referred to here as pHW) plasmid backbone[15] using iVEC, which involves mixing the *Dpn*I treated PCR reactions at 3:1 molar ratio of insert/vector prior to transformation.[59] The gene encoding sfGFP[37] was synthesized together with different combinations of the *lac* operators (pHW-sfGFP, pHW/O_{123}-sfGFP, pHW/O_{12}-GFP, pHW/O_{13}-sfGFP, and pHW/O_3-sfGFP) and/or the *E. coli* *rrnB* gene terminators (pHW/T1T2-sfGFP and pHW/O_{123}T1T2-sfGFP) and cloned into the *Sna*BI/*Nae*I sites of the pHW plasmid (GenScript USA). The avian N1 (1999 NA; GenBank ID: CY016957) and the HA (H1-BR18 and the H6) gene segments together with their 5′ and 3′ UTR's were cloned into the modified pHW plasmids by replacement of the gene encoding sfGFP using iVEC.

Transformation and Colony Screening. Ligation reactions consisting of 1 μL of the PCR insert and vector mixtures were transformed into 50 μL of XL10-Gold cells per the manufacturer's instructions (Agilent) and cultured overnight at 37 °C on LB-agar ampicillin plates. Plates were imaged with an Azure C600 and 5−10 individual or pooled colonies were randomly selected for growth on a master plate and for direct colony screening by PCR. For screening, colonies were resuspended in 1× PCR reaction buffer (RB) (10× RB: 200 mM Tris-HCl pH 8.8, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1 mg/mL BSA and 1% Triton) for lysis and the DNA was amplified over 30 cycles using Pfu X7 DNA polymerase and a primer pair targeting the plasmid (pHW FWD screening primer) and the specific insert (NA/HA reverse primer). The amplified DNA was analyzed by agarose gel (0.8%) electrophoresis. Overnight liquid cultures (LB broth) were used to amplify the positive clones for additional studies including virus rescue. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen), and all constructs were sequenced prior to use (Macrogen).

Fluorescent Size Exclusion Chromatography. Plasmids containing the gene encoding sfGFP were transformed into XL10 Gold cells and amplified overnight in 10 mL LB broth cultures containing 100 μg/mL ampicillin. The following day, 1 mL of the overnight culture was sedimented (10,000*g*; 5 min) and the bacterial pellets were resuspended in 1 mL lysis buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$,

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200 μg/mL lysozyme, 1× EDTA-free protease inhibitors, spec DNase I), incubated for 30 min at room temperature and sonicated on ice (5 s × 6; amplitude 10%). The sonicated lysates were sedimented (6000*g*; 1 min) to remove insoluble debris and analyzed by FSEC using an Agilent 1260 prime HPLC equipped with an AdvanceBio SEC 300Å column and a fluorescent detector set at 486 nm excitation and 524 nm emission wavelengths. A protein standard (AdvanceBio SEC 300Å protein standard; Agilent) of known molecular weights was included in each run to estimate the molecular weight/stokes radius of the expressed sfGFP.

GFP Expression Analysis. HEK 293T/17 cells (CRL-11268) were cultured at 37 °C with 5% CO_2 and ∼95% humidity in DMEM containing 10% FBS and 100 U/mL P/S. For each transfection, ∼7.5 × 10^5 HEK cells in DMEM containing 10% FBS were seeded in a 12-well plate. When the wells reached 75−80% confluency, ∼24 h post seeding, 1.0 μg of each pHW plasmid encoding sfGFP was separately added to 100 μL of OMEM, mixed with 3 μL of TransIT-LT1 transfection reagent, and incubated for 30 min at room temperature before addition to a well containing the HEK cells. Live-cell imaging for GFP expression was performed ∼60 h post-transfection using a Keyence BZ-X810 fluorescence microscope with a 10× objective and a BZ-X GFP cube filter (470 nm excitation and 525 nm emission wavelengths). Image capture settings were fixed across the experiment. Post-imaging, the cells in each well were harvested in 1 mL 1× phosphate buffered saline pH 7.2 (PBS), sedimented (6000*g*; 1 min), and resuspended in 150 μL lysis buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 0.5% *n*-dodecyl-β-D-maltoside, and 1× EDTA-free protease inhibitors). The lysed samples were sedimented (6000*g*; 1 min) to obtain a post-nuclear supernatant. GFP relative fluorescence units (RFU's) in each post-nuclear supernatant (100 μL) were measured in a 96-well low protein binding black clear bottom plate (Corning) on a Cytation 5 (Biotek) plate reader with 485 nm excitation and 528 nm emission wavelengths.

Viral RG. Madin−Darby canine kidney 2 (MDCK.2; CRL-2936) cells and HEK 293T/17 cells were cultured at 37 °C with 5% CO_2 and ∼95% humidity in DMEM containing 10% FBS and 100 U/mL P/S. Reassortant viruses were created by 8-plasmid RG in T25 flasks using the indicated NA, or NA and HA pair, and the complimentary seven, or six, gene segments of WSN or PR8 as previously described.[60] For each virus, ∼1.5 × 10^6 MDCK.2 cells in OMEM containing 10% FBS were seeded in a T25 flask and allowed to adhere for 45 min. During this period, the eight RG plasmids (1.5 μg of each) were added to 750 μL of OMEM, mixed with 24 μL of TransIT-LT1 transfection reagent, and incubated 20 min at room temperature. A 750 μL suspension of 293T/17 cells (∼3 × 10^6 cells/mL) in serum-free OMEM was added to each transfection mixture and incubated for 10 min at room temperature before addition to the T25 flask containing the MDCK.2 cells. At ∼24 h post-transfection, the media in each flask was replaced with 3.5 mL of DMEM containing 0.1% FBS, 0.3% BSA, 4 μg/mL TPCK trypsin, 1% P/S, and 1% L-glutamine. Following transfection NA activity and HAU measurements were taken at the indicated time points until viral harvest. Rescued viruses in the culture medium were harvested 72−96 h post-transfection, clarified by sedimentation (2000*g*; 5 min), and passaged in SPF eggs.

Viral Passaging in SPF Chicken Eggs. Initial passages (E1) were carried out by inoculating 9−11 day old embryonic SPF chicken eggs with 100 μL of the rescued virus either undiluted (PR8) or diluted 1/10 in PBS (WSN). Eggs were incubated for 3 days at 33 °C and placed at 4 °C for 2 h prior to harvesting. Allantoic fluid was harvested individually from each egg and clarified by sedimentation (2000*g*; 5 min). NA activity and HAU measurements were taken prior to combining each viral harvest for storage at −80 °C or viral purification.

Viral Isolation. Viruses in allantoic fluid were isolated by sedimentation (100,000*g*; 45 min) at 4 °C through a sucrose cushion (25% w/v sucrose, PBS pH 7.2 and 1 mM $CaCl_2$) equal to 12.5% of the sample volume. The supernatant was discarded, the sedimented virions were resuspended in 250 μL PBS pH 7.2 containing 1 mM $CaCl_2$ and the total protein concentration was determined using a BCA protein assay kit (Pierce). All isolated viruses were adjusted to a concentration of ∼500 μg/mL using PBS pH 7.2 containing 1 mM $CaCl_2$ prior to analysis on a 4−12% SDS-PAGE gel.

NA Activity, HAU, and Viral Titer Measurements. NA activity measurements were performed in 96-well low protein binding black clear bottom plates. Reactions were initiated by bringing up each sample (50 μL viral cell-culture medium or 10 μL allantoic fluid) to a volume of 200 μL with 37 °C RB (0.1 M KH_2PO_4 pH 6.0 and 1 mM $CaCl_2$) and 5 μL of 2 mM MUNANA. The fluorescence was measured on a Cytation 5 plate reader at 37 °C for 10 min using 30 s intervals and a 365 nm excitation wavelength and a 450 nm emission wavelength. Final activities were determined based on the slopes of the early linear region of the RFU versus time graph.

HAU titers were determined by a two-fold serial dilution in 96-well plates using a sample volume of 50 μL and PBS. Following the dilution, 50 μL of 0.5% TRBCs were added to each well and the plate was incubated 30 min at room temperature. HAU titers were determined as the last well where agglutination was observed. Median tissue culture infectious doses ($TCID_{50}$) per milliliter and median egg infectious doses (EID_{50}) per milliliter were calculated using 100 μL inoculums of MDCK cells and SPF eggs as previously described.[61] MDCK cell cytopathic effects and egg infections were verified by the presence of NA activity.

SDS-PAGE, Coomassie Staining. Purified virions equal to ∼5 μg of total viral protein were mixed with 2× sample buffer. Samples were heated at 50 °C for 10 min and resolved on a 4−12% polyacrylamide Tris-glycine SDS-PAGE wedge gel. Gels were stained with simple blue and imaged with an Azure C600.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00358.

Example sequence chromatograms of the mutations introduced to the NA and HA genes during plasmid propagation in *E. coli*, sequences and position of the regulatory elements introduced into the pHW plasmid, and the primer sequences used for cloning and PCR screening (PDF)

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Author Contributions

T.M., J.G., and R.D. conceived the study. T.M and L.K. performed the experiments. T.M., L.K., A.K., J.G., and R.D. analyzed the data. A.K. and J.G. provided reagents and expertise. T.M. assembled the figures and wrote the first draft of the manuscript. R.D. edited the manuscript with input from all authors, funded, and supervised the study.

Notes

The authors declare the following competing financial interest(s): A provisional patent application for the approach described in this study has been submitted.

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