Title
Gammaherpesviral Gene Expression and Virion Composition Are Broadly Controlled by Accelerated mRNA Degradation

Permalink
https://escholarship.org/uc/item/2jx143g9

Journal
PLoS Pathogens, 10(1)

ISSN
1553-7366

Authors
Abernathy, E
Clyde, K
Yeasmin, R
et al.

Publication Date
2014

DOI
10.1371/journal.ppat.1003882

Peer reviewed
**Abstract**

Lytic gammaherpesvirus infection restricts host gene expression by promoting widespread degradation of cytoplasmic mRNA through the activity of the viral endonuclease SOX. Though generally assumed to be selective for cellular transcripts, the extent to which SOX impacts viral mRNA stability has remained unknown. We addressed this issue using the model murine gammaherpesvirus MHV68 and, unexpectedly, found that all stages of viral gene expression are controlled through mRNA degradation. Using both comprehensive RNA expression profiling and half-life studies we reveal that the levels of the majority of viral mRNAs but not noncoding RNAs are tempered by MHV68 SOX (muSOX) activity. The targeting of viral mRNA by muSOX is functionally significant, as it impacts intracellular viral protein abundance and progeny virion composition. In the absence of muSOX-imposed gene expression control the viral particles display increased cell surface binding and entry as well as enhanced immediate early gene expression. These phenotypes culminate in a viral replication defect in multiple cell types as well as in vivo, highlighting the importance of maintaining the appropriate balance of viral RNA during gammaherpesviral infection. This is the first example of a virus that fails to broadly discriminate between cellular and viral transcripts during host shut off and instead uses the targeting of viral messages to fine-tune overall gene expression.


**Editor:** Linda Faye van Dyk, University of Colorado Denver School of Medicine, United States of America

**Received:** September 20, 2013; **Accepted:** November 26, 2013; **Published:** January 16, 2014

**Copyright:** © 2014 Abernathy et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This research was supported by grants R01 CA136367, R01 CA16056, a Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Disease award to BG, NIGMS 8P41GM103481 grant award to AB, and an American Cancer Research Scholar Grant, RSG-11-160-01-MPC award to LTK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: glaunsinger@berkeley.edu

**Introduction**

Viruses use a variety of mechanisms to dampen host gene expression, including inhibiting cap-dependent translation, transcription, splicing, and promoting host mRNA degradation. The presumed viral benefits of this ‘host shutoff’ phenotype include reduced competition for gene expression machinery and resources, as well as impaired immune responses through decreasing host factors involved in sensing infection. The importance of this phenotype in vivo has been directly confirmed for both alpha- and gammaherpesviruses, where host shutoff mutants exhibit defects in immune evasion (in the case of the herpes simplex viruses HSV-1 and HSV-2), viral trafficking, and latency establishment [1–3].

In all cases studied to date, viral transcripts largely escape the effects of host shutoff, thus affording them a competitive expression advantage. For example, poliovirus inhibits cap-dependent translation by cleaving eIF4G, thus enhancing translation of viral mRNAs that contain an internal ribosome entry site (IRES) but not a 5’ cap [4–6]. One mechanism of HSV-induced host shutoff involves altering phosphorylation of SR proteins to inhibit spliceosome assembly and block the biogenesis of nascent host mRNAs, the vast majority of which contain introns [7]. In contrast, HSV mRNAs are largely unspliced, enabling them to circumvent this block and, furthermore, are preferentially exported to the cytoplasm by the ICP27 protein [8,9]. HSV-1 also promotes endonucleolytic cleavage of host mRNAs through its virally encoded ribonuclease vhs, which is packaged into viral particles and can thus impact host gene expression immediately after viral entry [2,10]. Although HSV-1 mRNAs can be degraded by vhs in the absence of infection, recent data suggest that vhs is regulated by other viral factors in a manner that restricts its activity against viral RNA, particularly during delayed early and late gene expression [11,12]. SARS coronavirus also causes host shutoff by promoting endonucleolytic cleavage of cellular mRNAs, but its viral mRNAs bear a protective 5’ leader sequence that prevents their cleavage [13].

Similar to alphaherpesviruses and SARS coronavirus, gammaherpesviruses promote host shutoff by inducing widespread cellular mRNA degradation [14,15]. This viral subfamily includes the oncogenic human pathogens Kaposi’s sarcoma-associated...
Author Summary

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nucl ease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.

Viral Transcripts Are Controlled by Degradation

Many viruses restrict host gene expression during infection, presumably to provide a competitive expression advantage to viral transcripts. Not surprisingly, viruses that induce this ‘host shutoff’ phenotype therefore generally possess mechanisms to selectively spare viral genes. Gammaherpesviruses promote host shutoff by inducing widespread mRNA degradation, a process initiated by the viral SOX nuclelease. However, the effect of SOX on viral mRNA during infection was unknown. Here, we reveal that during infection with the murine gammaherpesvirus MHV68, the majority of viral transcripts of all kinetic classes are broadly down regulated through the activity of the MHV68 SOX protein (muSOX). We further demonstrate that in the absence of muSOX-induced control of viral mRNA abundance, viral protein levels increase, thereby affecting the composition of progeny viral particles. Altered virion composition directly impacts early events such as entry and induction of lytic gene expression in subsequent rounds of replication. Furthermore, decreasing both virus and host gene expression via global mRNA degradation is critical for viral replication in a cell type specific manner both in vitro and in vivo. This is the first example of a eukaryotic virus whose host shutoff mechanism similarly tempers viral gene expression, and highlights the degree to which gammaherpesviral gene expression must be fine tuned to ensure replicative success.
Figure 1. Expression of the majority of viral mRNAs is dampened during host shutoff. (A) For viral genes, the log2 fold change in expression upon infection with WT compared to ΔHS MHV68 expression was plotted and data points were colored to indicate the adjusted P values, with green points indicating positive log2 fold change with P value < 0.05 and red indicating negative log2 fold change with P value < 0.05. (B) RT-qPCR was used to validate selective viral transcripts. RNA was isolated at 24 hpi from NIH 3T3 cells infected with MR or ΔHS MHV68 at an MOI of 5. Transcript levels were normalized to 18 s and ΔHS levels set to 1. (C–F) mRNA half-life analyses were conducted by infecting NIH 3T3 cells with MR or ΔHS MHV68 at an MOI of 5. At 18 hpi, 2 μg of Actinomycin D was added to block transcription and RNA was harvested at the indicated times thereafter. RT-qPCR was performed with ORF-specific primers and probes to determine mRNA levels. The black dotted line indicates the best-fit line for the ΔHS virus, and the grey solid line indicates the best-fit line for the MR virus. doi:10.1371/journal.ppat.1003882.g001
3T3 cells with MR or ΔHS virus. The decay rate of each mRNA was calculated following addition of actinomycin D (ActD) to halt transcription at 18 hpi. In all cases, the ΔHS mutation led to a significant increase in transcript stability, indicating they are directly targeted for degradation by μSOX (Figure 1C–F). Thus, host shutoff is not restricted to cellular mRNAs, but also broadly impacts viral mRNA abundance during gammaherpesvirus infection.

Noncoding RNAs are enriched in the escapee population

Although the majority of MHV68 transcripts appear subject to degradation during host shutoff, a subset may escape this fate (Figure 1A, green bars). For example, levels of the M1 and M2 mRNAs are higher in MR infected cells relative to ΔHS infection as measured both by microarray analysis and RT-qPCR (Figure 2A). Interestingly, many of the other putative escapes are candidate or confirmed non-coding RNAs (ncRNAs). Indeed, analysis of the viral mRNA and ncRNA distribution revealed that most of the viral mRNAs fall under the μSOX-susceptible category, whereas the population of μSOX escapes is strongly enriched for ncRNAs (Figure 2B). This observation correlates well with previous data indicating that the gammaherpesvirus SOX proteins preferentially target translationally competent cellular mRNAs.

To confirm that noncoding transcripts are not depleted by μSOX, we compared the abundance of seven of the RNA polymerase III (Pol III) transcribed viral tRNAs during infection with MR or ΔHS virus at 24 hpi by RT-qPCR (Figure 2C–D). In contrast to the viral ORF54 mRNA, none of the tRNA levels were decreased during a MR infection relative to the infection with the ΔHS virus. We next examined several of the Expressed Genomic Regions (EGRs), which are RNA polymerase II (Pol II) transcripts distributed throughout the MHV68 genome that lack apparent coding capacity [23]. Similar to the viral tRNAs and in accordance with the microarray data, qPCR analysis indicated that several of the EGRs escape downregulation by μSOX, a finding confirmed by half-life analysis for EGRs 9, 15, and 26 (Figure 1A, 2E–H). Some EGRs, such as EGR1, were modestly or markedly upregulated during a MR infection, though this was due to a secondary transcriptional increase rather than enhanced RNA stability (data not shown). We noted that not all of the EGRs escape the effects of host shutoff, as EGRs 24, 27, and 29 were decreased during a MR relative to ΔHS MHV68 infection (Figure 2E). Given that the EGRs have yet to be functionally characterized, it is possible that those that are decreased in the presence of active μSOX are not truly noncoding, or may possess certain mRNA-like features. Collectively, however, these data suggest that μSOX preferentially targets viral mRNAs for degradation, while many viral ncRNAs are unaffected (or upregulated) during host shutoff.

RNA degradation alters intracellular viral protein levels and virion composition

We reasoned that if μSOX-induced depletion of viral mRNAs was important for regulating viral gene expression, then the differences in mRNA levels should lead to corresponding alterations in viral protein abundance. Protein levels were therefore measured for viral factors with available antibodies by Western blotting lysates of NIH 3T3 cells infected with MR or ΔHS MHV68. Indeed, in accordance with the mRNA abundance data, ΔHS infected cells contained higher protein levels of ORF51 (gp150), ORF8 (gB), ORF45, ORF49 (μSOX), ORF49, and ORF72 (β-Cyclin) relative to cells infected with MR virus (Figure 3A). Not all of the mRNA abundance changes resulted in altered protein levels however, as ORF4 (gp70) and ORF65 (M9) proteins accumulated to similar levels during MR and ΔHS infections even though their transcripts were targeted by μSOX.

These results indicate that in most cases, μSOX targeting of viral mRNAs regulates protein abundance during MR infection.

How might the broad μSOX-driven reduction in viral gene expression impact the outcome of infection? One possibility is that increased abundance of select viral proteins in the absence of functional μSOX might lead to alterations in viral particle composition, for example through elevated concentrations of envelope or tegument proteins. In particular, it has been shown that tegument composition can be influenced by intracellular protein concentration [24]. We therefore analyzed the composition of virions purified from MR or ΔHS infected cells by mass spectrometry (MS). Figure 3B shows the relative abundance of proteins detected in both MR and ΔHS virions from two MS runs (several proteins were detected in only 1 sample and thus were excluded from the comparative analysis; a full list of MS results are provided in Table S1). To ensure that differences were not due to unequal viral particle numbers between samples, peptide counts were first normalized to the major capsid protein ORF25, as capsid composition is geometrically constrained and thus should not be influenced by intracellular protein abundance. We then performed a secondary normalization against genome number (Table 1). Among the viral proteins available for comparison, there was a 2-3-fold increase in the abundance of select tegument (ORFs 45, 49, and 75c) and envelope (ORF8; gB) proteins in ΔHS virions relative to MR virions (Figure 3B). As expected, the levels of the minor capsid protein ORF65 and minor scaffolding protein ORF17 remained unchanged, validating the normalization strategy. Tegument proteins ORF75b and ORF52 appeared unchanged as well, indicating that not all components of the virion were altered. To confirm these data and compare the abundance of additional virion components not detected in the MS, purified viral particles were resolved by SDS-PAGE and Western blotted with antibodies against ORFs 4, 8, 45, 49, and 65. Indeed, tegument proteins ORF45 and ORF49 and the glycoprotein ORF8 (gB) accumulated to higher levels in ΔHS virions (Figure 3C). In contrast, we observed no differences in the relative abundance of the ORF65 minor capsid protein or ORF4 (gp70).

To confirm that altered virion composition in the absence of host shutoff was not restricted to virions derived from 3T3 cells, we compared the levels of the μSOX-impacted tegument proteins ORF45 and ORF49 by Western blot of virions purified from infected MEFs, and observed a similar increase in their levels in the ΔHS virus (Figure S3). Thus, in the absence of μSOX-induced mRNA degradation, the abundance of several viral proteins increases, resulting in altered composition of progeny virions.

Altered virion composition leads to enhanced cell surface binding and entry

We next sought to determine whether the differences in MR and ΔHS MHV68 particles resulted in any functional distinctions during a de novo infection. Given that viral envelope glycoproteins are involved in cell surface binding and internalization, we hypothesized that increased glycoprotein concentrations such as those we observed for gB might influence these events. We first measured viral attachment to NIH 3T3 cells and to the dendritic cell line DC2.4 by incubating them with MR or ΔHS MHV68 for 90 min at 4°C to allow attachment but prevent uptake, then measuring the relative level of attached virions by qPCR for the viral genome (Figure 4A). Indeed, there was a marked (10–15 fold) increase in ΔHS virus binding relative to MR binding in both cells.
Figure 2. Noncoding RNAs are enriched in the escapee population. (A) RT-qPCR on viral transcripts M1 and M2. RNA was harvested from infected NIH 3T3 cells at 24 hpi. Viral transcripts were normalized to 18 s and ΔHS levels set to 1. (B) The array data was used to determine what percentage of viral noncoding RNAs (ncRNAs) are upregulated during a MR infection (70%), and what percentage of viral mRNAs are downregulated (83.3%). (C) Schematic of the left end of the MHV68 genome, including the 8 viral tRNAs, Expressed Genomic Region (EGR 1), and ORFs M1 and M2. (D and E) RT-qPCR on viral ncRNA was done by harvesting RNA from infected NIH 3T3 cells at 24 hpi. cDNA was made using transcript specific reverse primers and each transcript was normalized to 18 s and ΔHS levels set to 1. ORF54 was used as a control to show downregulation. 3–5 independent RT-qPCRs were done for each transcript. (F–H) EGR half-life analyses were conducted by infecting NIH 3T3 cells with MR or ΔHS MHV68 at an MOI of 5. At 18 hpi, 2 ug of Actinomycin D was added to block transcription and RNA was harvested at the indicated times thereafter. RT-qPCR was performed with EGR-specific primers to determine transcript levels. The black dotted line indicates the best-fit line for the ΔHS virus, and the grey solid line indicates the best-fit line for the MR virus.

doi:10.1371/journal.ppat.1003882.g002
types (Figure 4A). We observed similar results when we quantified viral entry by incubating at 37°C for 90 min, acid stripping, and measuring intracellular viral particles by qPCR, indicating that the bound particles were successfully internalized (Figure 4B). This increase in binding and entry of ΔHS virions was not a secondary consequence of excess defective particles, as we observed no significant differences in the particle:PFU ratio between the WT, MR and ΔHS viruses (Figure S4). We conclude that the altered virion composition of ΔHS directly influences the first step of the viral lifecycle.

Failure to degrade viral mRNAs leads to enhanced lytic cycle entry

We next examined whether the altered virion composition influenced progression of the MHV68 lifestyle post entry. In particular, tegument proteins are deposited directly into newly infected cells and thus are poised to have an early impact on the course of infection. A key early outcome of a herpesvirus infection is whether the incoming virus establishes latency or progresses to lytic replication, with latency generally favored upon gammaherpesvirus infection in vivo and in cultured cells. Although MHV68 is atypical in that cell culture (unlike in vivo) it defaults to lytic replication, we previously observed that not all infected cells immediately enter the lytic cycle and a larger proportion of NIH 3T3 cells displayed a lytic marker (muSOX) upon infection with the ΔHS virus compared to the MR virus [1]. To determine whether the enhanced lytic entry phenotype of ΔHS MHV68 was specific to NIH3T3 cells or represented a more general effect, we measured the proportion of infected cells expressing the lytic protein ORF65 (M9) in both NIH 3T3 cells and mouse embryo fibroblasts (MEFs) at 18 hpi (Figure 5A). M9 was used as a lytic marker because its levels are not influenced by muSOX activity (see Figure 3A). Both viruses constitutively express GFP, which serves as a marker of infected cells. Indeed, in both NIH 3T3 and MEF cells 82.4% and 84.3%, respectively, of the GFP+ infected cells were also M9+ during infection with ΔHS virus. However, only 46.6% and 50.9% of NIH 3T3 and MEF cells infected with the MR virus were double positive (Figure 5A). Thus, RNA degradation by muSOX tempers viral lytic cycle entry during a de novo infection.

We hypothesized that the increased number of ΔHS infected cells directly entering the lytic cycle might be a consequence of enhanced activity of RTA, the major lytic transactivator. Although RTA is not a component of MHV68 particles, it is an IE gene whose activity is influenced by tegument proteins [25,26], some of which are over represented in ΔHS virions (Figure 3B–C). We assessed RTA activity at early times post infection by measuring levels of the RTA-responsive transcripts ORF 50 (RTA), ORF 57, and ORF 6 by RT-qPCR (Figure 5B–D). All three RTA-responsive transcripts were induced to significantly higher levels at 4 hpi (p-value<0.007) after infection with the ΔHS virus as compared to the MR virus (Figure 5C).
Table 1. Normalization strategy for mass spectrometry results.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MR ORF/capsid</th>
<th>Genome #</th>
<th>ΔHS ORF/capsid</th>
<th>Genome #</th>
</tr>
</thead>
<tbody>
<tr>
<td>capsid 25</td>
<td>1.22E-06</td>
<td>-</td>
<td>6.31E-07</td>
<td>-</td>
</tr>
<tr>
<td>ORF75c</td>
<td>7.52E-07</td>
<td>0.618955524</td>
<td>1.47E-08</td>
<td>0.589510263</td>
</tr>
<tr>
<td>ORF75b</td>
<td>5.57E-07</td>
<td>0.457868722</td>
<td>1.09E-08</td>
<td>0.343556856</td>
</tr>
<tr>
<td>gB</td>
<td>1.85E-07</td>
<td>0.151811516</td>
<td>3.61E-09</td>
<td>0.218056541</td>
</tr>
<tr>
<td>gM</td>
<td>2.96E-07</td>
<td>0.243808397</td>
<td>5.79E-09</td>
<td>0.265301148</td>
</tr>
<tr>
<td>ORF29</td>
<td>2.25E-07</td>
<td>0.185355195</td>
<td>4.40E-09</td>
<td>0.080678019</td>
</tr>
<tr>
<td>ORF59</td>
<td>8.43E-08</td>
<td>0.069367842</td>
<td>1.65E-09</td>
<td>0.221416547</td>
</tr>
<tr>
<td>capsid 25</td>
<td>1.57E-06</td>
<td>-</td>
<td>1.33E-06</td>
<td>-</td>
</tr>
<tr>
<td>ORF62</td>
<td>7.81E-07</td>
<td>0.496152539</td>
<td>5.38E-08</td>
<td>0.45300884</td>
</tr>
<tr>
<td>ORF52</td>
<td>2.51E-06</td>
<td>1.595944443</td>
<td>1.73E-07</td>
<td>0.896746041</td>
</tr>
<tr>
<td>ORF65</td>
<td>1.30E-06</td>
<td>0.823241982</td>
<td>8.93E-08</td>
<td>0.501103815</td>
</tr>
<tr>
<td>ORF59</td>
<td>2.05E-07</td>
<td>0.130477608</td>
<td>1.42E-08</td>
<td>0.555948069</td>
</tr>
<tr>
<td>ORF17</td>
<td>3.73E-07</td>
<td>0.236822723</td>
<td>2.57E-08</td>
<td>0.108114721</td>
</tr>
<tr>
<td>ORF45</td>
<td>2.56E-07</td>
<td>0.162454517</td>
<td>1.76E-08</td>
<td>0.296639457</td>
</tr>
</tbody>
</table>

Top portion of the table are proteins identified in both ΔHS and MR samples for the first mass spectrometry (MS) run. Lower portion of the table is from the second MS run. The peptide count was normalized to major capsid protein ORF 25, and subsequently normalized to genome number, as determined by qPCR.

aGenome number for first MS run.
bGenome number for second MS run.
doi:10.1371/journal.ppat.1003882.t001

Figure 4. Altered virion composition leads to enhanced cell surface binding and entry. (A) Schematic showing cells were infected at an MOI of 5 at 4°C to allow viral binding, but prevent uptake. Both NIH 3T3 cells and DC2.4 cells were infected with MR or ΔHS MHV68. Cells were washed 4X with PBS at 90 min post infection, DNA was isolated, and qPCR used to quantify relative DNA levels by normalizing gB to GAPDH levels and setting MR levels to 1. (B) Cells were infected at an MOI of 5 at 37°C to allow uptake of virions. At 90 min post infection, the viral particles not internalized were stripped from the surface by the addition of 40 mM citric acid for 5 minutes and washed 4X with PBS. DNA was isolated and qPCR used to quantify relative DNA levels of internalized virus. Each graph represents 3 independent experiments.
doi:10.1371/journal.ppat.1003882.g004
compared to the MR virus. A similar trend was observed at 6 and 8 hpi, although the differences at these time points were not statistically significant. Importantly, the levels of the cellular GAPDH mRNA remained unchanged between 2–8 hpi but, as expected, decreased at 24 hpi during host shutoff (Figure 5E), which generally starts 10–18 hpi [15]. This confirmed that the effects on viral transcript accumulation manifested prior to the onset of muSOX-induced mRNA degradation, and therefore must

Figure 5. Failure to degrade viral mRNAs leads to enhanced lytic cycle entry. (A) Shown is the percent of lytic-expressing infected NIH 3T3 or MEF cells. Cells were infected at an MOI of 5 with GFP-BAC MHV68 MR or ΔHS and were analyzed at 18 hpi for GFP and M9 expression by immunofluorescence using anti-M9 antibodies. 5 fields of view from three independent experiments were counted and the percentage of GFP+M9+ cells calculated. ** Indicates p-value<0.01, determined by student t-test. (B–D) To measure levels of RTA-responsive transcripts after infection with MR or ΔHS MHV68, NIH 3T3 cells were infected at an MOI of 5 and RNA harvested at indicated times post infection. RT-qPCR was used to quantify relative levels of ORF 50 (B), ORF 57 (C), and ORF 6 (D). (E) GAPDH levels were measured to show that host shutoff had not yet initiated at the 8 hpi time point.

doi:10.1371/journal.ppat.1003882.g005
derive from differences in the incoming viral particles. Thus, RTA activity is stimulated shortly after infection with the DHS virus, likely due to higher levels of RTA activators within the virion itself.

muSOX-induced mRNA degradation is important for viral amplification in a cell type specific manner in vitro and in vivo

Given the enhanced viral gene expression and lytic cycle entry phenotypes of the DHS virus, it might be predicted that this mutant virus would replicate more efficiently, as has been observed for MHV68 engineered to constitutively express RTA [27,28]. However, DHS was shown to have no growth advantage over MR in NIH 3T3 cells and, furthermore, in mice it displayed defects in trafficking from the lung to distal sites, as well as severely decreased abundance in splenocytes at 2 weeks post infection during peak latency establishment [1]. These observations suggest that there may be cell type specific replication defects associated with impaired muSOX RNA degradation activity that manifest subsequent to the viral entry and gene expression enhancements observed above. We therefore compared the replication rates of the MR and DHS viruses in NIH 3T3, MEF, and DC2.4 cells using multi-step growth curves. As reported previously, the MR and DHS viruses grew to similar titers in NIH 3T3 cells (Figure 6A). However, DHS displayed moderately delayed kinetics in MEF cells, resulting in a 2 log defect at 5 dpi (Figure 6B) and severely delayed kinetics in DC2.4 cells, resulting in a 5 log defect at 5 dpi (Figure 6C). The DC2.4 result is notable given that MHV68 infects and traffics through dendritic cells in mice [29]. Thus, although the DHS virus binds and enters these cells more efficiently than MR, its failure to control gene expression through muSOX activity results in a downstream amplification defect.

B cells are key sites of viral replication and latency establishment in vivo, however these cells cannot be readily infected in culture. We therefore sought to monitor viral replication in these cells in infected mice. MHV68 infection via the intraperitoneal route (i.p.) leads to lytic replication in macrophages and B cells in the spleen that peaks at 10 dpi, whereupon the lytically infected cells are largely cleared prior to the onset of peak latency at 18 dpi [30,31]. We therefore measured viral load in splenocytes of mice infected i.p. with each virus at 10 dpi during the lytic replication phase, and found significantly reduced viral titers with DHS compared to MR (Figure 6D). These data indicate that inhibition of muSOX-induced RNA degradation restricts MHV68 amplification in a cell type specific manner both in vitro and in vivo.

Discussion

Our findings demonstrate that the majority of gammaherpesviral mRNAs are targeted by the muSOX nuclease and thus reveal...
These effects manifested as a consequence of alterations in virion composition imposed during the prior round of replication. There is evidence suggesting that extensive protein-protein interactions dictate tegument composition and deleting certain proteins can alter this balance [24,43,44]. Furthermore, pseudorabies virions lacking select tegument proteins accumulate actin in the viral particles, suggesting there is a critical mass that is filled in relation to protein abundance in an infected cell [24,45]. These findings and others, together with our observations, indicate that tegumentation is a highly orchestrated process regulated through specific protein interactions as well as by protein abundance, which can be at least partially controlled at the level of viral mRNA stability. We anticipate that in addition to the protein composition changes examined herein, there are presumably increased levels of viral and perhaps cellular mRNAs packaged into the ΔHS virions that may also impact early events in the viral lifecycle. Indeed, it has been previously observed that RNAs can be packaged into herpesviral particles in a manner largely linked to their intracellular abundance [46,47].

In addition to potential structural roles in the virion, tegument proteins can manipulate the cellular environment immediately after their release and thus are thought to play key roles in facilitating infection [48]. For example, immune evasion is partly mediated through the activities of KSHV ORF56 and its homologs, which inhibit the type 1 IFN response [49], as well as HSV-1 UL41, which downregulates MHCII on the cell surface [50]. Other tegument proteins are involved in augmenting early viral gene expression, such as HSV-1 VP16, HCMV pp71, and KSHV RTA, which act as lytic transactivators through coordination with other viral and cellular factors [37,39,51,52]. Unlike in KSHV, RTA is not packaged into the MHV68 virion, although it is expressed with immediate early kinetics and several tegument proteins facilitate lytic infection by boosting its activity. For example, MHV68 ORF49 interacts with PARP-1, a cellular inhibitor of RTA [25], while ORF75c degrades PML-NBs, which interfere with lytic cycle progression [26]. Our observation that cells infected with the ΔHS virus displayed enhanced RTA activity shortly after infection confirms that failure to moderate viral protein levels through mRNA degradation has marked consequences for subsequent rounds of infection, including preferential or enhanced immediate early lytic gene expression.

MHV68 infects a variety of cell types in vitro with varying outcomes, including efficient lytic replication in fibroblasts, delayed lytic entry in dendritic cells, and primarily latent or abortive lytic infection in macrophages [33,54]. In vivo, evidence suggests that epithelial cells are lyrically infected, while dendritic cells, macrophages, and B cells mainly support latency after an initial burst of lytic infection to amplify the virus and seed a population of infected cells [31,55–57]. Previously, we reported that the MHV68 ΔHS virus exhibits dramatically reduced viral loads at 17 dpi during peak latency [1]. Here, we found that the ΔHS virus also exhibits cell type specific defects during the lytic amplification stage both in vitro and in cultured cells. Thus, despite the enhanced lytic gene expression phenotype of the ΔHS virus, one or more subsequent steps in the replication cycle are impaired in certain cell types. Though the basis for the cell autonomous replication defect remains unknown, the reduced amplification in vitro could manifest in part through impaired immune evasion, for example as a consequence of elevated viral antigen levels or abortive infection. One possibility is that the innate immune sensing and response mechanisms are differentially tuned in a cell type specific manner, such that failure to degrade viral and host mRNAs triggers a more robust antiviral response in certain cell types. Additionally, we have noted that the WT MHV68 lifecycle
Viral Transcripts Are Controlled by Degradation

progresses more slowly in dendritic cells relative to 3T3 cells or MEFs (unpublished observations), perhaps making these cells more impacted by the increased accumulation of viral mRNAs in the ΔHS infection.

Our data challenge the assumption that viruses that dampen host gene expression do so categorically to provide a competitive advantage to viral gene expression. Instead, we reveal that degradation of viral transcripts during lytic gammaherpesviral infection is integral to establishing the appropriate balance of viral factors necessary for replication and optimal virion composition. Clearly, degradation of viral mRNAs is not absolute and mechanisms may exist to temper the effects of muSOX to ensure adequate accumulation of viral transcripts. For example, it has been shown that KSHV ORF57 can stabilize viral RNA in the nucleus and cytoplasm [58, 59]. Additionally, viral mRNAs may contain regulatory sequences that help modulate their stability. Deciphering how the many layers of gene expression regulation are coordinated during infection remains an important future challenge.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of California Berkeley (approval number R292). All animals were anesthetized prior to infection with isoflurane, and all efforts were made to minimize suffering.

Cells, viruses, and infections

NIH 3T3, NIH 3T12, Vero, DC2.4, and MEF cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The green fluorescent protein (GFP)-expressing MHV68 bacterial artificial chromosome (BAC) has been described elsewhere [60], and the R443I host shutoff mutant was previously generated by allelic exchange as previously described [1]. BAC-derived MHV68 virus was produced by transfecting BAC DNA into NIH 3T3 cells using SuperFect (Qiagen). Virus was then amplified in NIH 3T12 cells and titered by plaque assay on NIH 3T3 cells. Before infecting mice, the loxP-flanked BAC vector sequence was removed by passaging the virus through Vero cells expressing Cre recombinase (kindly provided by Dr. Samuel Speck, Emory University).

Tiled microarray hybridization and analysis

Array data was derived from two independent biological replicates of each infection condition. Custom MHV68 tiled arrays in the 4 by 44,000 format were designed as described previously [36]. 1 μg of RNA was reverse transcribed using an oligo(dT) promoter-primer, subjected to linear amplification and Cy3 or Cy5 labeling using an Agilent Quick Amp Labeling kit. Adenovirus spike-in controls were added to each labeling reaction to allow normalization per the two-color spike-in kit instructions (Agilent). The Cy3-labeled reference RNA derived from an independent infection of NIH 3T3 cells at 8 hpi with WT MHV68 was generated, purified, fragmented, and then hybridized in parallel with the Cy3-labeled sample RNA at 65°C for 17 h. Microarrays were scanned with Agilent Scanner Control software (version 7.0), and hybridization signal intensities were quantified using Agilent Feature Extraction software (version 9.5).

Raw data were processed by subtracting the median background signals from the mean signals in the Agilent feature extraction file and then normalized by multiplying the log2 values of the probe intensities by the spike-in scale factor for each array. The scaling factor was calculated from the linear fit of the spike-ins versus their concentration. The normalized log2 Cy3-labeled reference RNA signal was subtracted from the normalized log2 Cy5-labeled experimental sample RNA signal for each probe. The expression value of each viral ORF was calculated as the median of the normalized signal of all tiling probes enclosed inside the ORF. Quality control analyses indicated strong correlation between the biological replicates (Figure S1). Differentially expressed genes were identified after performing empirical Bayes moderated t-statistics using the Bioconductor LIMMA package (version 3.12.1) [61].

Nucleic acid isolation and measurement

RNA was isolated using the Zymo Mini RNA II Isolation Kit (Zymo Research), treated with Turbo DNase (Ambion) to remove genomic DNA contamination, and reverse transcribed using AMV RT (Promega) with oligo(dT) and 18 s specific primers. For nRNA analysis, transcript specific reverse primers were used instead of oligo(dT) during cDNA synthesis. cDNA levels were then quantified using DyNAamo color flash SYBR green master mix, ROX passive reference dye (Thermo Scientific), and transcript specific primers (listed in Table S2). Transcript levels were normalized to 18 s. Viral genomes were quantified by isolating DNA using the DNeasy Blood and Tissue kit (Qiagen), and using 10-fold dilutions of 1 ng BAC DNA to generate a standard curve. For mRNA half-life analyses, at 18 hpi 2 μg of actinomycin D (Sigma) was added to halt transcription, and RNA was isolated at the indicated time points for quantification by RT-qPCR using TaqMan reagents (Applied Biosystems). Primers used to detect ORF54 (probe TCACACCTATATCTGTCCAACCCAGCGAA) and ORF55 (probe CCAACCTTTTGCCACGCAGC) are listed in Table S2. Final primer concentrations of 900 nM and probe concentrations of 250 nM were used. ORF57 and ORF8 primers and probes were used as described previously [1, 62]. GAPDH levels were measured using the TaqMan Rodent GAPDH Control and Ribosomal RNA Control reagents (Applied Biosystems). The data were plotted relative to the t = 0 time point which was set to 1.

Virion isolation and mass spectrometry

Viral supernatants were collected 7 dpi from 6 plates (15 cm) of NIH 3T3 or MEF cells infected at an MOI of 0.5 and centrifuged at 4,000 rpm for 15 min to pellet cellular debris, then subsequently centrifuged over a 20% sucrose cushion at 24,000 rpm for 1 h to pellet the virus. The pellet was resuspended in 200 μl PBS and centrifuged through a 10–60% continuous sucrose gradient at 24,000 rpm for 1 h. Fractions (1 ml) were collected, and DNA was extracted from a portion of each fraction using the DNeasy Blood and Tissue kit (Qiagen) to quantify viral DNA by qPCR using g-specific primers. Fractions enriched in viral genomes were pooled, pelleted by centrifugation at 24,000 rpm for 1 h, and resuspended in 100 μl of lysis buffer [50 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS] for Western blots or 50 μl of ammonium bicarbonate (25 mM) for mass spectrometry.

Western blots

Cell lysates were prepared in lysis buffer and quantified by Bradford assay. Equivalent amounts of each sample were resolved
by SDS-PAGE and Western blotted with the following anti-
MHV68 primary antibodies: hybridoma supernatants T1A1 anti-
gp130, MG-2C10 anti-gB, and 9c7 anti-gp70, diluted 1:10 (kindly
provided by Phillip Stevenson, University of Cambridge); anti-M9,
anti-ORF26, and anti-ORF45, diluted 1:500 (kindly provided by
Ren Sun, UCL); anti-ORF72, diluted 1:500 (kindly provided by
Linda van Dyk, University of Colorado, Denver); anti-ORF49,
diluted 1:500 (kindly provided by Moon Jung Song, Korea
University). Primary antibodies were followed by HRP-conjugated
good anti-mouse or goat anti-rabbit secondary antibodies (Southern
Biotechnology, 1:5000).

Viral binding and entry assays

NIH 3T3 or DC2.4 cells were infected at 4°C (to measure binding)
or 37°C (to measure entry) for 90 min at an MOI of 5. For the binding assay, cells were then washed 4X with ice-cold
PBS, scraped, and DNA isolated by DNeasy Blood and Tissue
kit (Qiagen). For the entry assay, cells were washed 2X with PBS,
and 0.5 ml of citric acid [135 mM NaCl, 10 mM KCl, 40 mM citric acid, pH 3] was added for 5 minutes at RT to
strip off remaining cell surface-bound viral particles. Cells were
washed twice more with PBS, scraped, and DNA was isolated.
Relative genome levels were quantified by qPCR with gB-
specific primers.

Immunofluorescence assays

NIH 3T3 or MEF cells were grown on coverslips and infected with GFP-BAC MHV68 at MOI of 5. At 24 hpi, coverslips were
washed with PBS and cells were fixed in 4% formaldehyde for 30 min at RT. Cells were then permeabilized in 1% Triton-X-100
and 0.1% sodium citrate in PBS for 10 min, and incubated with anti-M9 antibodies at 1:500 in 10% goat serum at 37°C. After
1 hour, coverslips were washed 3X in PBS and incubated with
good anti-rabbit AlexaFluor546 secondary antibody at 1:1500
(Invitrogen). Coverslips were washed 3X in PBS and mounted in
DAPI-containing Vectashield mounting medium (VectorLabs) to
stain cell nuclei.

Growth curves and in vivo experiments

For multi-step growth curves, 1.5×10^5 NIH 3T3, MEF, or
DC2.4 cells were infected with MHV68 at MOI of 0.05 and both
supernatant and cells were harvested at 0, 1, 2, 3, 4, and 5 dpi and
frozen. Samples were freeze-thawed once before titering by plaque
assay on NIH 3T3 cells.

For in vivo experiments, female C57BL/6j mice were
obtained from the Jackson Laboratory (Bar Harbor, ME) and
infected when 4–6 weeks old through injection of 1×10^5 pfu of
virus in 0.2 ml PBS into the peritoneal cavity. Spleens were
harvested at 10 dpi, and DNA isolated and quantified by
qPCR, using gB-specific primers to determine relative levels of
viral genomes.

Supplemental methods

For quality control, the scaled log2 Cy3-labeled experimental
cRNA probe values and the scaled log2 Cy5-labeled reference
cRNA probe values for each WT and ΔH5 replicate were
determined as described above and then compared in box plots
using R package ‘graphics’ ignoring outliers [63]. In addition, the
scaled log2 Cy5-labeled reference cRNA signal was subtracted
from the scaled log2 Cy3-labeled experimental sample cRNA
signal for each probe. The normalized probe values for the WT
and ΔH5 replicates were analyzed by scatter plot. Linear model
fit of the scatter plots was performed using ‘lm’ from R package
‘stats’ [63].

Supporting Information

Figure S1 Consistency between array replicates. (A) Box
and whisker plots of the scaled log2 Cy3-labeled experimental
cRNA probe values (green) and the scaled log2 Cy5-labeled
reference cRNA probe values (red). (B) Scatter plot and linear
model fit (black line) of the normalized probe values of the WT
replicates (r^2 = 0.9622) and ΔH5 replicates (r^2 = 0.9095).
(PDF)

Figure S2 Viral transcripts are degraded during
MHV68 infection independent of cell type. DC2.4 (A) or
MEF (B) cells were infected with MR or ΔH5 MHV68 at MOI of
5 and RNA was harvested 48 hpi (for DC2.4) or 24 hpi (for
MEFs). Viral transcript levels were determined by RT-qPCR,
and normalized to 18S rRNA. At least 2 independent biological
replicates were performed for each cell type. (EPS)

Figure S3 Altered virion composition is independent of
cell type. (A) ΔH5s were infected with MR or ΔH5 MHV68 for
5 days, whereupon virions were isolated by sucrose gradient
centrifugation and abundance of the virion tegument proteins
ORF45 and ORF49, and the capsid protein ORF26 were
determined by Western blot. Protein abundance was quantified
graphed relative to the ORF26 capsid protein. At least 2
independent replicates of each Western blot were analyzed.
(EPS)

Figure S4 The particle to PFU ratio is similar between
viruses. (A) Particle number was determined by isolating DNA
from viral stocks and performing qPCR using gB primers. PFU
was determined by plaque assay. At least 5 viral stocks from
ΔH5 and MR, and 3 from WT were assessed for both particle
number and PFU. Differences are not statistically significant.
(EPS)

Table S1 Complete list of proteins identified through
mass spectrometry. All proteins identified by MS, accession
number, molecular weight, and peptide count and normalized to
protein length and total counts. *First MS run. All proteins listed
were identified in either ΔH5 or MR samples. **Second MS run.
All proteins listed were identified in either ΔH5 or MR samples.
(DOCX)

Table S2 All primers used in this study. All forward and
reverse primers used for each ORF or noncoding RNA.
(DOCX)

Acknowledgments

We are grateful to the many investigators who provided reagents for this
study, including Drs. Phillip Stevenson, Ren Sun, Linda van Dyk, Moon
Jung Song, and Samuel Speck. We are grateful to Kathy Li for analyzing the
mass spectrometry data. L.T.K. and R.Y. thank Hong Wang for
technical support. We would also like to thank members of the
Glausinger, Krug, and Cossoy laboratories for helpful discussions and
critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: EA KC BG. Performed the
experiments: EA KC LTK. Analyzed the data: EA KC LTK Ry BG.
Contributed reagents/materials/analysis tools: AB RY LTK LC. Wrote
the paper: EA BG.

PLOS Pathogens | www.plospathogens.org
12 January 2014 | Volume 10 | Issue 1 | e1003882
References


PLOS Pathogens | www.plospathogens.org 13 January 2014 | Volume 10 | Issue 1 | e1003882
Viral Transcripts Are Controlled by Degradation