Viral Nucleases Induce an mRNA Degradation-Transcription Feedback Loop in Mammalian Cells

Graphical Abstract

Highlights

- Herpesvirus-induced cytoplasmic mRNA decay causes transcriptional alterations
- The mRNA decay-transcription feedback mechanism requires cellular decay factors
- Herpesviral genes escape mRNA degradation-induced transcriptional repression

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In Brief

Gamma-herpesviruses encode an endonuclease that induces cellular mRNA degradation. Abernathy et al. show that viral nuclease-triggered mRNA decay leads to repression of cellular transcription and decreased RNA Polymerase II (RNAPII) recruitment. RNAPII-transcribed viral genes escape this transcriptional repression, highlighting how viruses use this mRNA decay-transcription feedback mechanism for their benefit.

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Viral Nucleases Induce an mRNA Degradation-Transcription Feedback Loop in Mammalian Cells

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SUMMARY

Gamma-herpesviruses encode a cytoplasmic mRNA-targeting endonuclease, SOX, that cleaves most cellular mRNAs. Cleaved fragments are subsequently degraded by the cellular 5′-3′ mRNA exonuclease Xrn1, thereby suppressing cellular gene expression and facilitating viral evasion of host defenses. We reveal that mammalian cells respond to this widespread cytoplasmic mRNA decay by altering RNA Polymerase II (RNAPII) transcription in the nucleus. Measuring RNAPII recruitment to promoters and nascent mRNA synthesis revealed that the majority of affected genes are transcriptionally repressed in SOX-expressing cells. The transcriptional feedback does not occur in response to the initial viral endonuclease-induced cleavage, but instead to degradation of the cleaved fragments by cellular exonucleases. In particular, Xrn1 catalytic activity is required for transcriptional repression. Notably, viral mRNA transcription escapes decay-induced repression, and this escape requires Xrn1. Collectively, these results indicate that mRNA decay rates impact transcription and that gamma-herpesviruses use this feedback mechanism to facilitate viral gene expression.

INTRODUCTION

Viruses are extensively integrated into the cellular gene expression network, having evolved strategies to alter or co-opt machinery involved in the stages of transcription and RNA fate through translation and protein turnover. As such, they have served as valuable tools to dissect the pathways that govern cellular gene expression. Though gene expression is often considered in terms of a unidirectional flow of discrete events, there are an increasing number of examples of how these basic stages are interconnected (Braun and Young, 2014; Huch and Nissan, 2014). Such feedback mechanisms may enable cells to maintain homeostasis or mount appropriate responses during periods of perturbation. Viral infections represent a significant stress for the cell and thus are likely to alter or stimulate crosstalk between components of the gene expression cascade.

Recent work has revealed that a feedback loop exists between mRNA synthesis and degradation in S. cerevisiae (Haimovich et al., 2013; Sun et al., 2013). One of the key proteins involved in linking mRNA decay to transcription is the 5′-3′ mRNA exonuclease Xrn1, which is the primary exonuclease involved in cytoplasmic mRNA degradation in Drosophila, yeast, and mammals (Nagarajan et al., 2013). However, although the data are consistent that Xrn1 deletion impacts mRNA synthesis in yeast, reports differ both as to the specific requirement for Xrn1, as well as whether it serves as a direct or indirect transcriptional regulator (Haimovich et al., 2013; Sun et al., 2013).

Whether similar cytoplasmic mRNA decay-transcription feedback mechanisms are operational in higher eukaryotes such as mammals remains unknown. Furthermore, how enhanced mRNA degradation might signal through such a feedback loop is an open question and one that is difficult to address through mutant studies. In this regard, several mammalian viruses rapidly accelerate cytoplasmic mRNA degradation through the combined activity of virally encoded mRNA-targeting endonucleases and mammalian Xrn1 and thus could provide insight into these questions (Gaglia et al., 2012). Members of the alpha- and gamma-herpesvirus subfamilies, as well as influenza A virus (IAV) and SARS coronavirus (SCoV), all encode viral proteins that target mRNAs for endonucleolytic cleavage (Glaunsinger and Ganem, 2004; Jagger et al., 2012; Kamitani et al., 2009; Kwong and Frenkel, 1987; Rowe et al., 2007). Though the viral proteins are not homologous, in all examined cases they bypass the rate-limiting deadenylation and decapping events by inducing internal cleavages in cytoplasmic mRNA, and then rely on the cellular mRNA decay machinery to degrade the cleaved mRNA fragments. For the alpha- and gamma-herpesviruses and SCoV, clearance of cleaved mRNAs requires Xrn1 (Covarrubias et al., 2011; Gaglia et al., 2012).

Here, by comparing the effects of gamma-herpesviruses that contain wild-type or inactivated mRNA-targeting nucleases, we reveal a direct connection between accelerated cytoplasmic mRNA decay and altered RNA Polymerase II (RNAPII) transcription in mammalian cells. However, contrary to what might be predicted based on observations in S. cerevisiae, we find that enhancing mRNA degradation leads predominantly to a decrease in RNAPII activity on cellular genes, although a subset of genes are transcriptionally upregulated. We show a central
role for cellular exonucleases including Xrn1 in this repression, indicating that Xrn1-linked transcriptional regulation is a feature conserved between S. cerevisiae and mammals. Furthermore, our findings support the conclusion that it is the act of mRNA degradation by cellular nucleases that is sensed and triggers transcriptional alterations, rather than secondary effects from stabilization of mRNAs encoding transcriptional regulators. Interestingly, viral transcription, which is also mediated by RNAPII, largely escapes transcriptional repression.

RESULTS

Enhanced mRNA Turnover in the Cytoplasm Suppresses RNAPII Transcription

Infection with murine gamma-herpesvirus 68 (MHV68) leads to widespread acceleration of mRNA decay in the cytoplasm that is initiated by mRNA cleavage by the viral endoribonuclease muSOX and completed by degradation of the cleaved fragments by the cellular 5′-3′ exoribonuclease Xrn1 (Covarrubias et al., 2009; Gaglia et al., 2012). A point mutation in the muSOX gene at position R443 (R443I; ΔHS) renders it defective for cleaving cytoplasmic RNAs, and thus infection with the ΔHS mutant virus does not broadly increase mRNA decay (Richner et al., 2011). We therefore queried how infection of NIH 3T3 cells with WT MHV68 versus the ΔHS mutant impacted rates of cellular mRNA transcription as measured by 4-thiouridine (4sU) pulse labeling. Just prior to harvesting, mock-infected or infected cells were incubated for 30 min with 4sU, which gets incorporated into actively transcribing mRNAs and can be subsequently coupled to HPDP-biotin and purified over magnetic streptavidin beads. Quantification by RT-qPCR of the housekeeping genes Gapdh, Rpl37, and ActB from purified 4sU-labeled RNA showed a significant transcriptional reduction during MHV68 infection compared to mock-infected cells (Figure 1A). No reduction in transcription was detected in cells infected with the ΔHS point mutant virus, suggesting that the transcriptional suppression observed during WT MHV68 infection was specifically linked to enhanced cytoplasmic mRNA decay (Figure 1A). We detected robust transcriptional activation during both MHV68 WT and ΔHS infection of the interferon-stimulated gene IFIH1 (Liu et al., 2012), indicating the 4sU assay accurately portrays transcription changes. We applied several tests to confirm that the abundance of 4sU-containing mRNA reflected nascent transcription rather than decay rates in the cytoplasm. First, we showed that 4sU-labeled mRNA remained largely confined to the nucleus at the time of harvest (Figure 1B). Second, we also quantified the 4sU-labeled RNA using primers within intronic sequences, confirming that the transcriptional repression observed during WT MHV68 infection was specifically linked to enhanced cytoplasmic mRNA decay (Figure 1A). We detected robust transcriptional activation during both MHV68 WT and ΔHS infection of the interferon-stimulated gene IFIH1 (Liu et al., 2012), indicating the 4sU assay accurately portrays transcription changes. We applied several tests to confirm that the abundance of 4sU-containing mRNA reflected nascent transcription rather than decay rates in the cytoplasm. First, we showed that 4sU-labeled mRNA remained largely confined to the nucleus at the time of harvest (Figure 1B). Second, we also quantified the 4sU-labeled RNA using primers within intronic sequences, confirming that the transcriptional repression was observed for pre-mRNA (Figure 1C). Finally, we observed similar transcriptional repression when applying only a 5-min 4sU pulse, and normalizing each sample to the number of cells harvested after 4sU addition confirmed nascent RNA levels are altered only during a WT infection (Figures S1A and S1B).

To test directly whether the transcriptional alterations that occurred during MHV68 infection were due to accelerated mRNA decay, we examined whether this phenotype could be
recapitulated upon expression of the viral endonuclease alone. HEK293T cells were transfected with plasmids expressing WT muSOX, the catalytically dead point mutant muSOX D219A, or the viral endonuclease vhs from herpes simplex virus (HSV-1). Although not homologous to muSOX, HSV-1 vhs is also a broad-acting, cytoplasmic, mRNA-specific endonuclease that engages Xrn1 to degrade the cleaved RNA fragments (Gaglia et al., 2012; Read, 2013). Similar to our results in infected cells, 4sU labeling showed a reduction of transcription of the housekeeping genes Gapdh, ActB, GusB, and eEF-1a in cells expressing the viral endonuclease alone. We also measured RNAPII occupancy at cellular promoters by chromatin immunoprecipitation (ChIP) assays and, in agreement with our 4sU labeling, observed a reduction in RNAPII occupancy in cells expressing vhs or muSOX but not muSOX D219A (Figure 1E).

Finally, we performed a similar set of experiments with the human gamma-herpesvirus Kaposi’s sarcoma-associated herpesvirus (KSHV). KSHV encodes a SOX gene that functions in a manner analogous to MHV68 muSOX (Covarrubias et al., 2009; Glaunsinger and Ganem, 2004). We engineered a P176S point mutation in the KSHV SOX gene, which, similar to the MHV68 ΔHS mutant, confers a specific mRNA degradation defect (Glaunsinger et al., 2005). We monitored mRNA degradation-induced transcriptional changes during the lytic KSHV replication cycle using iSLK renal carcinoma cells, which harbor a doxycycline (dox)-inducible version of the major lytic cycle trans-activator RTA and can be stimulated to replicate the virus upon treatment with dox and sodium butyrate (Myoung and Ganem, 2011). Measurement of transcription rates 48 hr post-lytic reactivation by 4sU labeling showed a specific transcriptional repression of the housekeeping genes Gapdh, ActB, GusB, and eEF-1a in cells containing WT KSHV but not the P176S mutant (Figure 1F). Collectively, these data suggest that virus-induced cytoplasmic mRNA degradation induces RNAPII transcriptional repression.

**Cellular Exonucleases Are Required for the mRNA Decay-Transcription Feedback Mechanism**

We next sought to determine what cellular factor(s) were required to activate the mRNA decay-induced transcriptional feedback mechanism. Given that Xrn1 degrades the mRNA fragments cleaved by the viral endonucleases, we reasoned that Xrn1 activity might be involved in the transcriptional response to mRNA degradation in mammalian cells. We generated HEK293T cells stably expressing dox-inducible Xrn1-targeting shRNAs. After Xrn1 knockdown by dox treatment for 4 days, the cells were transfected with plasmids expressing either WT or D219A muSOX, and RNAPII promoter occupancy was measured by ChIP. In control cells not treated with dox, we observed the expected reduced RNAPII occupancy at the Gapdh promoter in the presence of WT muSOX, but not the D219A catalytic mutant (Figure 2A). However, Xrn1 knockdown restored RNAPII occupancy at the Gapdh promoter in cells expressing muSOX to levels observed in cells expressing D219A. Importantly, in these experiments mRNAs are cleaved by muSOX regardless of Xrn1 levels. Furthermore, knockdown of Xrn1 in control cells lacking viral nuclease did not result in transcriptional changes by RNAPII ChIP at two cellular promoters (Figure 2B). Thus, it can be concluded that differences in transcription result from a mechanism to sense accelerated mRNA degradation, rather than secondary effects stemming from altered stability of mRNAs encoding transcriptional regulators.

To determine if this effect was specific to Xrn1, we also knocked down three other mammalian factors involved in basal mRNA decay, the 3'-5' exonuclease Dis3L2 and the deadenylases Ccr4 and Pan2 (Figures 2C and 2E). Although Xrn1 is the only mammalian exonuclease characterized as involved in the degradation of muSOX-cleaved mRNAs, it is likely that 3'-5' exonucleases also help degrade the cleavage fragments. Similar to the results with Xrn1, depletion of Dis3L2 restored RNAPII occupancy in muSOX-expressing cells to those of control cells (Figure 2C). No effect of Dis3L2 knockdown on RNAPII promoter recruitment was observed in the absence of the viral nuclease (Figure 2D). Finally, although knockdown of Ccr4 alone did not impact the reduction of RNAPII promoter occupancy in muSOX-expressing cells, co-depletion of both Ccr4 and Pan2 restored RNAPII occupancy at the Gapdh promoter (Figure 2E). Unlike our results with Xrn1 and Dis3L2, knockdown of the deadenylases led to an increase in RNAPII promoter occupancy even in the absence of muSOX (Figure 2F), suggesting that in uninfected cells, alterations in deadenylase activity are monitored and drive transcriptional feedback. These data indicate that multiple cellular exonucleases contribute to transcriptional feedback in mammalian cells. Furthermore, in the cases of Xrn1 and Dis3L2, it is their enhanced activity in the presence of widespread mRNA cleavage during infection that is sensed, rather than indirect effects stemming from altered basal mRNA decay.

**Xrn1 Catalytic Activity Is Required for Reduced RNAPII Transcription**

Because the role of Xrn1 in clearing muSOX-cleaved fragments is well established, we focused on this enzyme and applied a complementation assay to determine if Xrn1 catalytic activity was required for repression of cellular transcription. Cells expressing WT or D219A muSOX were knocked down for endogenous Xrn1 and complemented with plasmids expressing either WT Xrn1 or the catalytically dead mutant D208A (Jinek et al., 2011) (Figure 3A). RNAPII ChIP showed that introduction of WT but not D208A Xrn1 restored the degradation-induced transcriptional repression of the Gapdh promoter in WT muSOX-expressing cells (Figure 3B). This suggests that catalytic activity is required to induce repression of RNAPII transcription and is in agreement with findings in S. cerevisiae (Haimovich et al., 2013).

We next explored the possibility that Xrn1 might be directly acting to influence transcription, as has been suggested in yeast (Haimovich et al., 2013). To determine whether Xrn1 translocates to the nucleus in cells undergoing accelerated mRNA decay, we monitored Xrn1 localization during MHV68 infection by immunofluorescence assay (IFA). Although transiently expressed Xrn1 appears to be exclusively cytoplasmic (unpublished data), we observed endogenous Xrn1 in both the nucleus and cytoplasm in NIH 3T3 cells (Figure S2A). The IFA signal was specific for Xrn1, as pre-treatment of the cells with Xrn1-targeting siRNAs significantly decreased the staining in both the nucleus and the cytoplasm (Figure S2A). However, the nuclear-cytoplasmic distribution of Xrn1 was not altered during infection with WT
MHV68 or the ΔHS mutant (Figures S2B and S2C). We were also unable to detect enrichment of Xrn1 at transcriptionally impacted cellular promoters in ChIP assays (data not shown). These data suggest that it is the sensing of Xrn1 activity in the cytoplasm that leads to transcriptional alterations, rather than a cis-acting effect of Xrn1 on cellular promoters.

We next examined whether mRNA decay primarily impacted RNAPII promoter recruitment or elongation. WT MHV68 infection decreased RNAPII promoter occupancy both at the promoter as well as within the gene (Figure 3C), suggesting the transcriptional repression induced by WT MHV68 infection is at least partly due to reduced RNAPII recruitment. Furthermore, the ratio of total to serine-2 (Ser2)-phosphorylated RNAPII (a marker of elongating polymerase; Phattanai and Greenleaf, 2006) was unchanged in response to MHV68 infection of NIH 3T3 cells (Figures 3D and 3E). No alterations in RNAPII occupancy were observed in cells infected with the ΔHS MHV68 (Figure 3D).

We next calculated the transcription elongation rates at three cellular genes using the reversible RNAPII inhibitor 5,6-dichloro-benzimidazole 1-β-d-ribofuranoside (DRB) coupled with 4sU (Fuchs et al., 2014) to measure the speed of polymerase elongation between sites proximal and distal to each promoter (Figure 3E). We observed no significant differences between the elongation rates at the three cellular genes tested in cells infected with WT or ΔHS virus (Figure 3F). Although there was reduced elongation upon infection at the Gapdh gene, this was not linked to mRNA decay, as a similar decrease was observed in the ΔHS-infected cells. Collectively, these data are consistent with RNAPII recruitment being the primary target of mRNA decay-linked transcriptional repression.
Figure 3. Xrn1 Catalytic Activity Is Required for Reduced RNAPII Transcription

(A) Diagram showing the complementation assay procedure. HEK293T cells with dox-inducible Xrn1 were mock or dox-treated for 4 days, whereupon cells were transfected with plasmids expressing WT or the catalytically dead D208A Xrn1 mutant, as well as with muSOX or muSOX D219A.

(B) Following the above procedure, ChIP and qPCR were performed to measure RNAPII recruitment to the human Gapdh promoter.

(C) NIH 3T3 cells were infected with WT or ΔHS MHV68 for 24 hr. RNAPII recruitment to the Rpl37 and ActB promoters and internal genes was measured by ChIP followed by qPCR.

(D) Ser2 ChIP was performed using the internal Rpl37 and ActB promoters. The level of Ser2-phosphorylated RNAPII was determined by dividing the Ser2 values over the total RNAPII within the same region of the gene.

(E) Diagram showing procedure for calculating transcription elongation rates. DRB was added to infected cells for 3 hr, then removed, and cells were 4sU-labeled. Conditions tested include no DRB treatment (NT), DRB without washout (DRB), 8 min 4sU after DRB washout (8 min), and 12 min 4sU after DRB washout (12 min).

(F) Relative kb/min calculated by normalizing RNA levels to NT and DRB and subtracting the amount of 4sU-labeled RNA at 10 kb (distal) at 12 min from the amount at 1 kb (proximal) at 8 min. Error bars represent the mean with SEM of ≥ 3 independently performed experiments. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.
Cellular Transcriptional Changes Occur throughout the mRNA Transcriptome

To determine the extent of transcriptional alterations that occur in response to accelerated cytoplasmic degradation, we sequenced libraries of 4sU-labeled RNA from mock-, WT-, or ΔHS MHV68-infected NIH 3T3 cells on the Illumina platform (Figure 4A). Relative to uninfected samples, WT MHV68 infection resulted in a $\geq 1.5$-fold transcriptional decrease of 9.25% of genes based on log2 fold change (Figure 4B, full list of genes in Table S1). Independent validations of 4sU-labeled mRNA levels by RT-qPCR confirmed the sequencing results for 12 out of 19 genes tested (Figures S3A and S3B). The seven genes in which the two assays were not in agreement showed transcriptional repression by RT-qPCR but not by 4sU-seq, perhaps suggesting that the 4sU-seq represents a conservative estimation of the breadth of degradation-induced transcriptional alterations.

Among the set of transcriptionally repressed genes during WT infection, 374 were categorized as statistically significant based on read counts and fold change. In contrast, only 38 genes were significantly reduced during a ΔHS infection, and among these, 32 overlapped with those in the WT infection samples. Thus, these overlapping genes are likely downregulated as a result of viral infection and are not specific to mRNA degradation (Figure 4C).

Gene ontology (GO) term-based analysis of the set of 342 genes that were transcriptionally repressed only during WT MHV68 infection yielded no clear links to specific biological processes (Table S2), suggesting that mRNA degradation-induced transcriptional repression is not restricted to specific functional classes of genes.

Unlike during WT infection, where transcriptional changes were more frequently repressive, the transcriptional changes that occurred during ΔHS infection were equally split between induced and repressed categories (3.19% versus 3.18%, respectively). In addition to the set of transcriptionally repressed genes, we also observed a subset of genes (6.87%) that showed a $\geq 1.5$-fold increase upon WT MHV68 infection (Figure 4B). Furthermore, a larger fraction of the significantly transcriptionally induced genes during a WT infection overlapped with those induced during ΔHS infection (32.6% of induced genes compared to 8.6% of reduced genes), suggesting that upregulation is less likely to be linked to mRNA degradation and more likely to be linked to viral infection (Figure 4C). Notably, among the set of 85 genes whose transcriptional induction was common to both WT and ΔHS infection, GO-term analyses returned a clear enrichment for genes involved in antiviral defense mechanisms and in nucleotide binding (Figure 4D). Although the significance of the latter remains to be determined, the induction of antiviral response factors would be a predicted transcriptional response to infection, independent of mRNA degradation.
Viral mRNAs Escape Degradation-Induced Transcriptional Repression

Herpesviral mRNAs are transcribed in the nucleus using the host machinery. We therefore analyzed the transcriptional changes that occurred at each of the viral genes in response to mRNA degradation during WT and ΔHS infection using the 4sU-seq dataset. Interestingly, viral genes largely escaped the transcriptional repression (Figure 5A). Independent validation experiments confirmed that even genes that appeared to undergo modest transcriptional repression during WT infection by 4sU-seq were unchanged or even slightly upregulated as measured by RT-qPCR of 4sU-labeled RNA isolated from cells infected with the WT or ΔHS MHV68. Results are normalized to 18S and ΔHS values are set to 1. (C) ChIP for total RNAPII was performed on NIH 3T3 cells infected with WT or ΔHS MHV68, and the % input values of the viral M1 and ORF8 promoters were compared. (D) iSLK cells latently infected with WT or P176S KSHV were reactivated for 48 hr with dox and sodium butyrate, then labeled with 4sU for 30 min prior to RNA isolation. RNA levels were compared by RT-qPCR for the indicated viral genes. Error bars represent the mean with SEM of ≥3 independently performed experiments.

Xrn1 Positively Influences Viral Transcription during Widespread mRNA Decay

Although the majority of MHV68 genes are susceptible to muSOX cleavage during infection, several viral transcripts appear to escape muSOX-mediated degradation (Abernathy et al., 2014). Two of these putative “escapees,” the viral ORF M1 (an RNAPII transcript) and the viral tRNA-like gene vttRNA1 (an RNAPIII transcript), exhibit enhanced steady-state expression during WT relative to ΔHS infection, perhaps due to increased transcription (Abernathy et al., 2014). To test whether muSOX-induced transcriptional feedback was responsible for their increased abundance during WT infection, we first confirmed that the M1 and vttRNA1 half-lives were not altered during a WT versus ΔHS MHV68 infection (Figure 6A). We then evaluated whether the transcriptional enhancement of these viral genes during WT MHV68 infection was linked to Xrn1 activity using the HEK293T cells expressing dox-inducible Xrn1-targeting shRNAs. Upon Xrn1 knockdown, M1 expression was significantly reduced during WT but not ΔHS MHV68 infection, and its expression was...
restored upon introduction of exogenous WT Xrn1 (Figure 6B). The requirement for Xrn1 appeared specific for RNAPII-driven transcription, as its depletion had no significant impact on expression of the RNAPIII-transcribed vtRNA1 (Figure 6C). RNAPII ChIP experiments confirmed that the reduction in M1 mRNA in the absence of Xrn1 was due to transcriptional repression (Figure 6D). We also observed reduced RNAPII occupancy upon Xrn1 depletion at ORF54, an MHV68 gene that is susceptible to cleavage by muSOX (Abernathy et al., 2014), indicating that the role of Xrn1 in promoting viral transcription is not limited to transcripts that escape degradation (Figure 6D). In each of these experiments, the requirement for Xrn1 was only observed during WT infection and not during infection with the ΔHS virus. We did not detect any binding of Xrn1 to viral promoters by ChIP (data not shown), suggesting that it likely indirectly impacts viral transcription in cells undergoing enhanced mRNA decay. Finally, depletion of the Ccr4 deadenylase did not alter M1 transcription during a WT or ΔHS infection, in agreement with its dispensability for the repression of cellular transcription when depleted in isolation (Figure 6E). Collectively, these data demonstrate that in contrast to its role in transcriptional repression of many cellular genes, Xrn1 activity during muSOX-induced cytoplasmic mRNA decay is required for robust transcription of viral genes.

**DISCUSSION**

Here we used virally encoded mRNA-targeting endonucleases to show that cytoplasmic mRNA degradation and nuclear RNAPII transcription are linked in mammalian cells. Accelerated mRNA degradation generally results in transcriptional repression of cellular genes, although there is a subset of genes that are induced. Our findings therefore suggest that mammalian cells have a mechanism to sense broad alterations in RNA degradation. It is not the initial cleavages by viral endonucleases that are detected, but rather the increased activity of cellular exonucleases involved in degrading the cleaved mRNA fragments that generates a transcriptional response. Several cellular exonucleases involved in basal mRNA decay appear central to the transcriptional feedback activated by enhanced mRNA degradation. Notably, enhanced Xrn1 activity appears to have opposing consequences for host and viral transcription, suggesting that herpesviruses have evolved to benefit from this intrinsic feedback mechanism.

Our findings have some clear parallels to gene expression feedback pathways recently described in yeast, although the mammalian response to accelerated mRNA decay does not result in the transcriptional “buffering” phenotype observed in *S. cerevisiae*. In yeast, reducing cytoplasmic mRNA decay through the deletion of components of the mRNA degradation machinery results in a compensatory decrease in RNAPII transcription rates (Haimovich et al., 2013; Sun et al., 2013). Conversely, an RNAPII mutant that exhibits ~3-fold-reduced mRNA synthesis rates displays decreased rates of mRNA turnover in the cytoplasm (Sun et al., 2012). Our data indicate that mammalian cells also possess a mechanism to sense overall mRNA abundance. However, accelerated cytoplasmic mRNA degradation in mammalian cells induces transcriptional alterations that are distinct from this buffering phenotype, in that the majority of mRNA degradation-induced changes involved transcriptional repression. Conversely, our observation that knockdown of the Ccr4 and Pan2 deadenylases increased RNAPII promoter occupancy in uninfected cells suggests that decreased cytoplasmic decay stimulates transcription in mammalian cells. Thus, the transcriptional feedback pathway in mammalian cells appears to operate in a different direction than in yeast. It is notable that in uninfected cells we did not
observe changes in RNAPII occupancy upon depletion of Xrn1 or Dis3L2, perhaps reflecting the ability of alternative exonucleases to compensate for their decreased activity. However, presumably because deadenylation is generally the first and rate-limiting step of basal mRNA decay, stalling that arm of the pathway is sufficient to activate the transcriptional response. Transcriptional repression could represent a way for the cell to conserve energy during stress, or may have evolved as a countermeasure to viral infection, as widespread mRNA decay is more likely to be linked to pathogenesis. If so, gamma-herpesviruses have developed a means to avoid this restriction.

While the subset of transcriptionally repressed genes identified by the 4sU-seq dataset appears smaller than anticipated if this response is global, these results likely represent a conservative estimation of the scope of affected genes. Several genes for which we detected robust decay-induced transcriptional repression by 4sU-RT-qPCR showed more modest effects (or appeared unchanged) in the 4sU-seq data. Possible explanations for this underrepresentation by the 4sU-seq pipeline include the high rates of duplication common to nascent RNA-seq, as well as possible overall lower RNA abundance in cells undergoing widespread mRNA degradation, both of which might mask differential expression. Indeed, when we normalized our 4sU-RT-qPCR data to cell number rather than RNA abundance, we observed an even more dramatic reduction in nascent RNA levels between mock and WT infection.

Our observations linking Xm1 activity in the cytoplasm to transcriptional alterations complements recent reports in yeast that document a role for Xm1 in the degradation-transcription feedback loop. Although there is a consensus that Xm1 is involved in transcription, whether it or the other cellular nucleases operate directly via promoter binding or indirectly by impacting the abundance of mRNAs encoding transcriptional regulators (or by another mechanism) remains to be resolved. Two studies demonstrate that yeast Xm1 can shuttle into the nucleus and bind cellular promoters to enhance transcription initiation and elongation (Haimovich et al., 2013; Medina et al., 2014). These same studies show that several other cellular decay factors also shuttle into the nucleus and rely on Xm1 catalytic activity for nuclear import (Haimovich et al., 2013). Another report instead suggests that Xm1 impacts transcription indirectly in yeast through degradation of mRNAs encoding transcriptional regulators (Sun et al., 2013), although how protein levels would function in transcription, and the cytoplasm, where they are implicated by the 4sU-seq pipeline in the absence of nuclear decay cleavage. However, the cleaved fragments will not be efficiently degraded. Thus, transcriptional changes we observed in mammalian cells should not be due to altered levels of transcriptional regulators, in agreement with the fact that we did not detect alterations in transcription in response to cellular exonuclease depletion in the absence of nuclear decay expression. Instead, we hypothesize that feedback between RNA decay and synthesis may instead be regulated by altered nuclear-cytoplasmic distribution of nucleic acid binding proteins in response to mRNA degradation. For example, yeast polymerase subunits Rpb4/7 shuttle between the nuclear, where they function in transcription, and the cytoplasm, where they are involved in mRNA decay and translation initiation (Harel-Sharvit et al., 2010; Lotan et al., 2007). It may be that factors classically linked to transcription, mRNA decay, and translation function to coordinate and integrate several cellular processes in response to pathogenic or environmental cues (Harel-Sharvit et al., 2010). We propose that this systemic interconnectedness is present in mammalian cells and that viral infections introduce perturbations to mRNA stability whose downstream consequences impact multiple cellular processes.

**EXPERIMENTAL PROCEDURES**

**Cells, Transfections, and Transductions**

NIH 3T3 and HEK293T cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells were transfected at 75%-90% confluence with polyethylenimine for 24 hr. Plasmids pCDNA3-muSOX, pCDNA3-muSOX.D219A, and pCDNA3-vhs have been described previously (Covarrubias et al., 2009; Glausinger and Ganem, 2004). Plasmid pFN21-Halo-Xrn1 was kindly provided by Carol Wilusz and subcloned into the pFN21 vector with a FLAG tag. The Xm1 D208A mutation was introduced by site-directed mutagenesis to generate FLAG-Xrn1.D208A.

HEK293T cells were transduced with TRIPZ inducible lentiviral shRNA constructs (Thermo Scientific) against Xm1 (clone ID: V2THS_89028), Dis3L2 (clone ID: V3THS_391760), or CNOT6 (clone ID: V2THS_262587). Cells were transfected with shRNA, pSAX2 (lentiviral packaging), and pMD2.G (lentiviral envelope) (Addgene) for 48 hr, whereupon the supernatant was passed through 0.45-μm filters, mixed with 8 μg/ml of polybrene, and spun onto a monolayer of HEK293T cells at 1,500 rpm for 1.5 hr. Fresh media was then added and the cells were incubated for 5-7 days in selection media containing 1 μg/ml puromycin. Cell lines were induced with 1 μg/ml doxycycline for 4-5 days, and knockdown efficiency was determined by western blot and RT-qPCR. Pan2 siRNA (CGGAAUCUCAAUUCCAGA Ut; Life Technologies, 113470) was transfected into cells using INTERFERin (PolyPlus) for 48 hr.

**Viruses and Infections**

The MHV68 bacterial artificial chromosome (BAC) has been described elsewhere (Adler et al., 2000), and the construction of the R443I muSOX mutant
were then aligned to the mouse genome (mm10) or to the murine viral genome adaptor and read quality trimming (Trim Galore, Babraham Institute). Reads on Illumina HiSeq 2500. Raw reads from the instrument were subjected to quantified by RT-qPCR. All qPCR results were normalized to 18S levels. All infected NIH 3T3 cells, and RNA was isolated at the indicated time points and buffer containing 10

The 4sU protocol continued as described above.

For fractionated 4sU assays, cells were labeled with 4sU as above. Cells were scraped and spun for 10 s at 4°C at max speed. Supernatant was removed and pellet was resuspended in 380 µl ice-cold hypotonic lysis buffer (HBL: 10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM MgCl₂, 0.3% NP-40, 10% glycerol). Cells were incubated on ice for 10 min, then vortexed and spun again. The supernatant was collected as the cytoplasmic fraction and the pellet was resuspended in HLB. The cells were washed with HLB 3x and the pellet (nuclear fraction) was resuspended in 1 ml TRizol and phenol-chloroform extracted. The cytoplasmic fraction was phenol-chloroform extracted and the 4sU protocol continued as described above.

4sU-DRB was performed by adding 100 µM DBR (Sigma) in DMSO for 3 h, washing 2x with PBS, and adding 4sU for 8 or 12 min. Relative kb/mn were calculated by normalizing each time point to the WT sample for mock, WT, and ΔHS infection, followed by a normalization to the DRB sample for mock, WT, and ΔHS to account for background. The number of kb between the HS infection, followed by a normalization to the DRB sample for mock, WT, and ΔHS to account for background. The number of kb between the

4sU Labeling

Cells were labeled with DMEM containing 500 µM 4sU (Sigma) for indicated times prior to isolating RNA with TRizol, followed by isopropanol precipitation. Total RNA (100 µg) was incubated in biotinylation buffer (10 mM Tris [pH 7.4], 1 mM EDTA) and 200 µg HDP-D-biotin (EZ-link HDP-biotin; Thermo Scientific) with constant rotation at room temperature for 1.5 h. RNA was then phenol-chloroform extracted and precipitated with isopropanol. The pellet was resuspended in DEPC-treated water and mixed with 50 µl Dynabeads MyOne streptavidin C1 (Invitrogen) that had been pre-washed twice with 1X wash buffer (100 mM Tris [pH 7.5], 10 mM EDTA, 1 mM NaCl, 0.1% Tween 20). Samples were rotated for 15 min at RT, then washed 3x with 65°C wash buffer and 3x with RT wash buffer. Samples were eluted with 100 µM DTT, and the RNA was precipitated with ethanol prior to RT-qPCR. All qPCR results were normalized to 18S levels and WT or vector control set to 1.

ΔHS was previously described (Richner et al., 2011). MHV68 was produced by transfecting NIH 3T3 cells with BAC DNA using SuperFect (QIAGEN). Virus was amplified in NIH 3T12 cells and titrated by plaque assay. Cells were infected with MHV68 at an MOI of 5 for 24 h unless otherwise noted. KSHV BAC mutagenesis has been described elsewhere (Brulois et al., 2012). The mutant BAC clone was sequenced to confirm the P176S mutation, once after cell line construction, and once after 2 weeks of cell maintenance.

KSHV reactivated by adding 1 µg/ml doxycycline and 1 µg/ml sodium butyrate for 48 h. Reactivation efficiency was determined by qPCR on isolated DNA and found to be equivalent between WT and P176S.

ChIP

ChIP has been described previously (Listerman et al., 2006), with the following modifications: chromatin was sheared using a Covaris sonicator for 30 rounds of 30 s pulses with 210 V. Chromatin (100 µg) was diluted in 400 µl ChIP dilution buffer containing 10 µg RNAPII antibody (N20-X, Santa Cruz) or Xrn1 (Sigma) and rotated overnight at 4°C. DNA was isolated after reversing the crosslinks using QIAgien PCR clean up kit prior to qPCR. Each sample was normalized to input.

RT-qPCR

RNA was treated with Turbo DNase (Ambion) and reverse transcribed using AMV RT (Promega) with random 9-mer primers. cDNA was quantified using iTaq Universal SYBR Master Mix (Bio-Rad) and transcript-specific primers. For RNA half-life analyses, 5 µg/ml actinomycin D (Sigma) was added to infected NIH 3T3 cells, and RNA was isolated at the indicated time points and quantified by RT-qPCR. All qPCR results were normalized to 18S levels. All primers used in this study are in Table S3.

4sU RNA-Sequencing

4sU labeled RNA, in duplicate for each sample, was enriched, precipitated, and ribosome depleted (Ribo-Zero). A whole RNA Illumina TruSeq library was then constructed, and 100-bp paired-end sequencing was performed on Illumina HiSeq 2500. Raw reads from the instrument were subjected to adaptor and read quality trimming (Trimm Galore, Babraham Institute). Reads were then aligned to the mouse genome (mm10) or to the murine viral genome using TopHat (Trapnell et al., 2009). Gene count tables for known mouse and viral genes were constructed from TopHat alignments using htsq-quant (Anders et al., 2015). DESeq2 (Love et al., 2014) was then used to estimate pairwise differentially expressed genes. False discovery rate of 10% and log2 ratio of ± 1 were used to filter differentially expressed genes.

GO-term analysis was performed using DAVID bioinformatics resources (v. 6.7) for the induced and reduced gene sets found in both WT- and ΔHS-infected cells (85 and 32 genes, respectively), as well as the genes reduced only during a WT infection (342 genes), and genes only induced during a WT infection (176 genes). A functional annotation chart was generated and sorted by Benjamini false discovery rate of ≤ 0.05.

Western Blots

Dox-inducible cell lines were treated with 1 µg/ml of dox for 5 days, and cell lysates were prepared with lysis buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.5% glycerol) and quantified by Bradford assay. Equivalent amounts of each sample were resolved by SDS-PAGE and western blotted with antibodies against Xrn1 (Bethyl; diluted 1:200), Dis3L2 (kindly provided by Torben Jensen; diluted 1:500), Ccr4 (diluted 1:1,000), Pol2 (diluted 1:1,000), and actin (diluted 1:200). Primary antibodies were followed by HRP-conjugated secondary antibodies (Southern Biotechnol, 1:5,000).

SUPPLEMENTAL INFORMATION

Raw data are available at the NCBI GEO database (GEO: GSE70481). The individual sample accession numbers are GEO: GSM1782681 (mock 1), GEO: GSM1782682 (mock 2), GEO: GSM1782683 (WT MHV68 1), GEO: GSM1782684 (WT MHV68 2), GEO: GSM1782685 (ΔHS MHV68 1), and GEO: GSM1782686 (ΔHS MHV68 2).

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