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14. ABSTRACT

This report outlines the progress made on our Exploration: Hypothesis Development Award within the past 12 months. Briefly, the goals of the proposal were to characterize the oncolytic capacity of Herpes simplex virus type 1 ICP0 mutants in prostate cancer cells given the relationship between ICP0 and two tumor suppressors, RNase L and PML, implicated in prostate cancer progression. Here, we report that ICP0 prevents an RNase L-independent rRNA degradation event in infected cells (appended manuscript). We also provide preliminary evidence that suggests that the ability of ICP0-null HSV-1 mutants to selectively kill prostate cancer cells correlates with a reduction in PML levels. Furthermore, we briefly discuss experiments to be completed within the remaining six months of this award.

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Introduction

Current cancer therapies often have limited efficacy and high toxicity and thus novel approaches to treatment are sought. Recent work has focused on viruses as potential anti-cancer agents [1, 2]. The study of viruses has greatly increased our knowledge of many basic principles of biology, including cell growth control and tumorigenesis, along with our understanding of how viruses exploit their host to enable their replication. With this greater comprehension comes the ability to manipulate viruses to target and lyse cancerous cells. Some viruses are naturally oncotropic while others are being engineered to selectively replicate in tumor cells. Oncolytic viruses obtain their specificity by exploiting cell surface or intracellular defects in gene expression during tumor evolution. Recent studies indicate that many such defects lie in interferon (IFN) pathways [3-6], given the potent antiproliferative activity of IFN and its downstream mediators [7, 8]. At least two tumor suppressors, RNase L and PML, are regulated by IFN and have been implicated in prostate cancer. Linkage analyses demonstrated that the hereditary prostate cancer locus, HPC1, mapped specifically to the *RNASEL* gene and segregated within families with the most severe cases of prostate cancer [9]. RNase L degrades both viral and cellular rRNA, leading to protein synthesis inhibition and apoptosis [10]. PML is a ubiquitously expressed nuclear phosphoprotein shown to block the initiation, promotion and progression of a variety of tumors [11]. PML concentrates in sub-nuclear structures called ND10 and suppresses cell growth by inducing cell cycle arrest or apoptosis. Although PML protein expression is reduced or abolished in many human cancers, tumor tissue microarrays showed the highest percentage of reduction in prostate adenocarcinomas [12]. Furthermore, loss of PML was associated with tumor progression in prostate, breast and CNS cancers. Given that PML and RNase L are well-characterized ISGs, it has been suggested that defects in pathways involving apoptosis and innate immunity may be critical for prostate cancer initiation [13]. The human herpesvirus HSV-1 was the first virus to be engineered for oncolytic virus therapy. We recently published that mutants of HSV-1 lacking the immediate early protein ICP0 demonstrate enhanced oncolytic capacity in a mouse model of breast adenocarcinoma [14]. Pertinent to this project, ICP0 degrades PML [15, 16] and blocks the degradation of rRNA (Appendix 1), leading to the hypothesis that disruption of anti-viral and anti-proliferative pathways in prostate cancer cells render them sensitive to ICP0-null HSV mutants.

Body

In our Statement of Work, we proposed four general aims to characterize prostate cancer cell oncolysis by Herpes simplex virus type 1 (HSV-1) ICP0 mutants based on previous work implicating the IFN regulated tumor suppressors RNase L and PML in prostate cancer progression. For each aim listed below, a summary of our research accomplishments will be given. A graduate student, Paul Sobol, was recruited in September 2004 to work on this project.

1. Determine the role of ICP0 in preventing 28S & 18S rRNA degradation and its relationship to RNase L.

We had previously observed that infection of cells with HSV-1 mutants bearing lesions in ICP0 resulted in rRNA degradation at late times of infection. Since RNase L mediates antiviral immunity by degrading rRNA and we have shown that ICP0 blocks IFN activity [17, 18], we investigated the relationship between RNase L and ICP0. As outlined in the attached manuscript (Appendix), we found that ICP0 blocks rRNA degradation in an RNase L-independent fashion. This manuscript has been accepted (pending modifications) to Journal of Virology.

2. Determine the oncolytic properties of ICP0 mutant viruses in normal and cancerous prostate cells.

We have screened established normal and metastatic prostate cell lines, including RWPE-1 (normal human prostate epithelial), PC3 (bone metastasis derived), DU145 (brain metastasis derived) and LNCaP (lymph node metastasis derived) for their susceptibility to oncolysis with a series of ICP0 mutant viruses (KM100, KM110 and KM120). As outlined in Figure 1, LNCaP are the most permissive to infection by these viruses. Of interest, only LNCaP is refractory to type I IFN [19], due to the lack of a critical component of the IFN signaling pathway [20], and is the most differentiated of the three cancer lines.

3. Correlate tumor cell permissiveness with RNase L and PML levels and activities.

Given that ICP0 appears to block rRNA degradation mediated by a ribonuclease distinct from RNase L, we have focused our studies on the tumor suppressor PML. We have preliminary data suggesting that decreased levels of PML correlate with increased susceptibility to oncolysis with HSV-1 mutants lacking ICP0 (Figure 2). In agreement with Figure 2, LNCaP demonstrate the greatest reduction in PML levels of the three prostate cancer cell lines. We are confirming these studies by looking at total cellular levels of PML by western blot analysis.

4. Develop a murine model of prostate cancer to test the oncolytic ability of ICP0 mutant viruses.

We have completed an *in vivo* assessment of the oncolytic capacity of the HSV-1 ICP0 mutant KM100 in a mouse model of breast adenocarcinoma [14]. These findings will serve as a platform for *in vivo* studies of prostate cancer oncolysis. Given that in the breast cancer model, infection with KM100 elicited a strong anti-tumor immune response and rendered mice resistant to subsequent tumor challenge, we chose to investigate an immunocompetent mouse model of prostate cancer (TRAMP model). We have ordered TRAMP-C1, -C2 and –C3 cells from the ATCC and will begin these studies shortly.

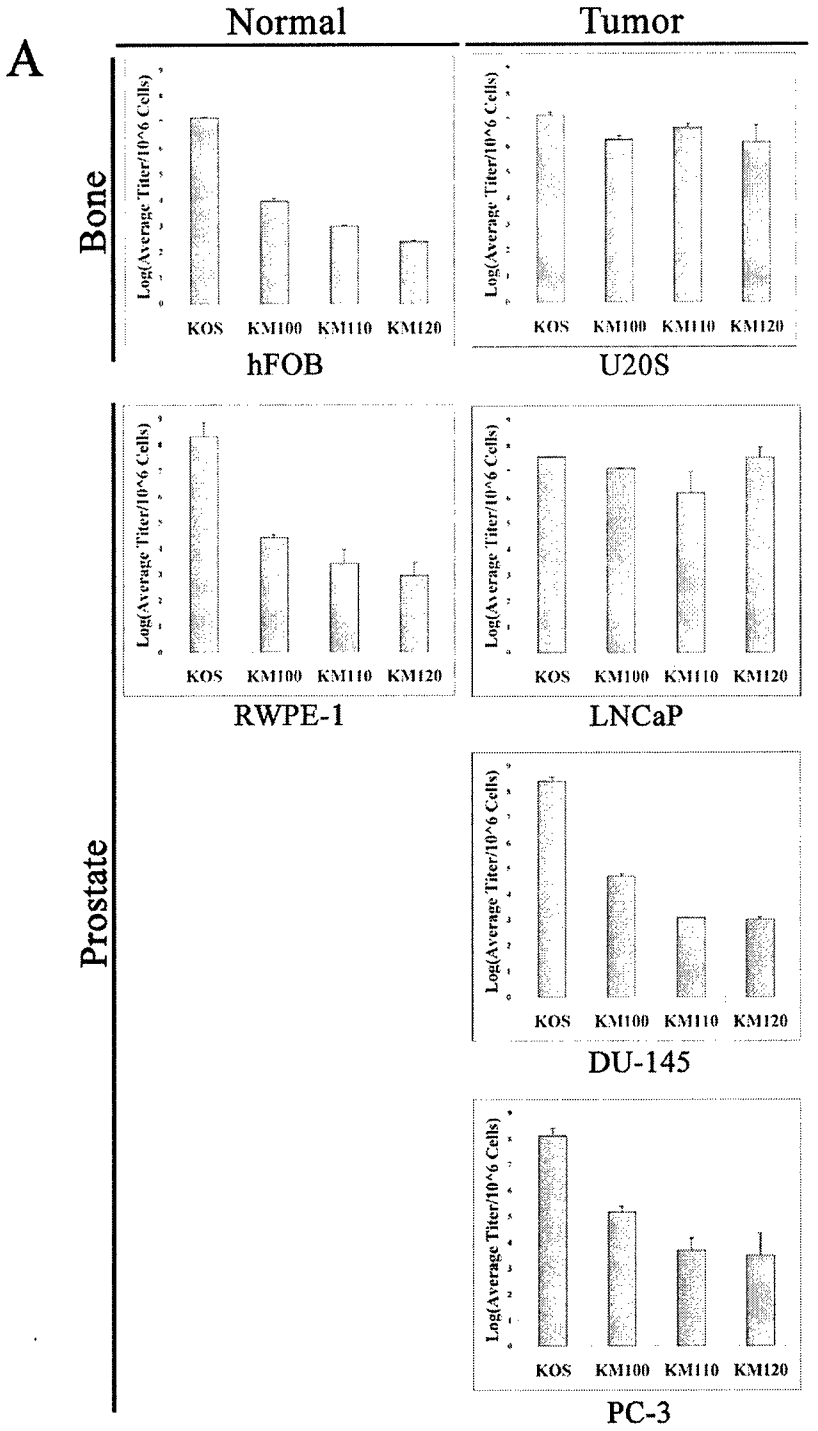

Figure 1. (A) Average viral titres per one million cells, adjusted to log scale. Cell lines tested were normal/tumor pairs for bone and prostate tissues.

B

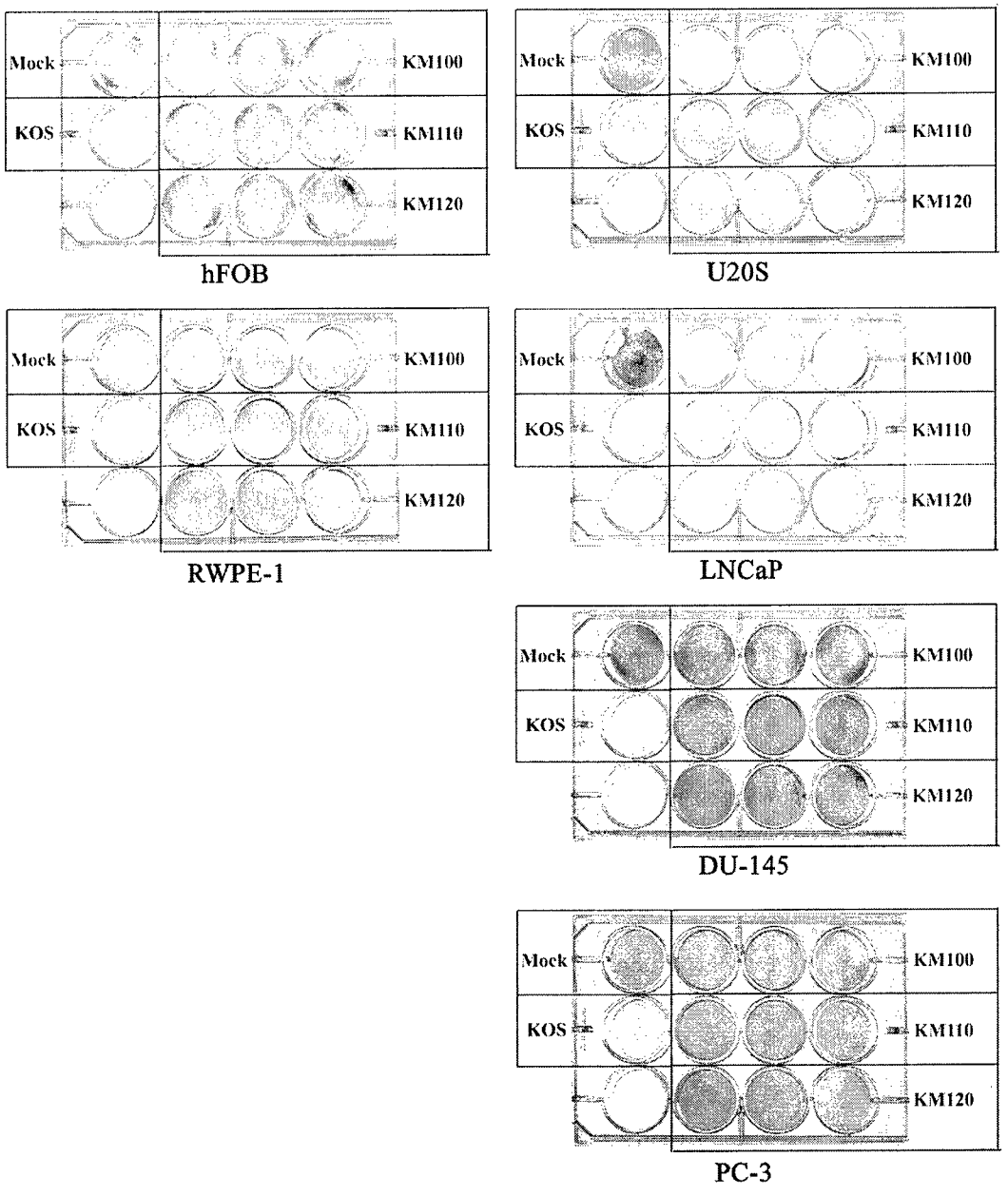

Figure 1. (B) Cytopathic effect (CPE) of ICP0-null mutant HSV on normal/tumor bone and prostate cell lines. Mock treatment (no virus) represents no CPE, whereas KOS infection represents a positive control for CPE.

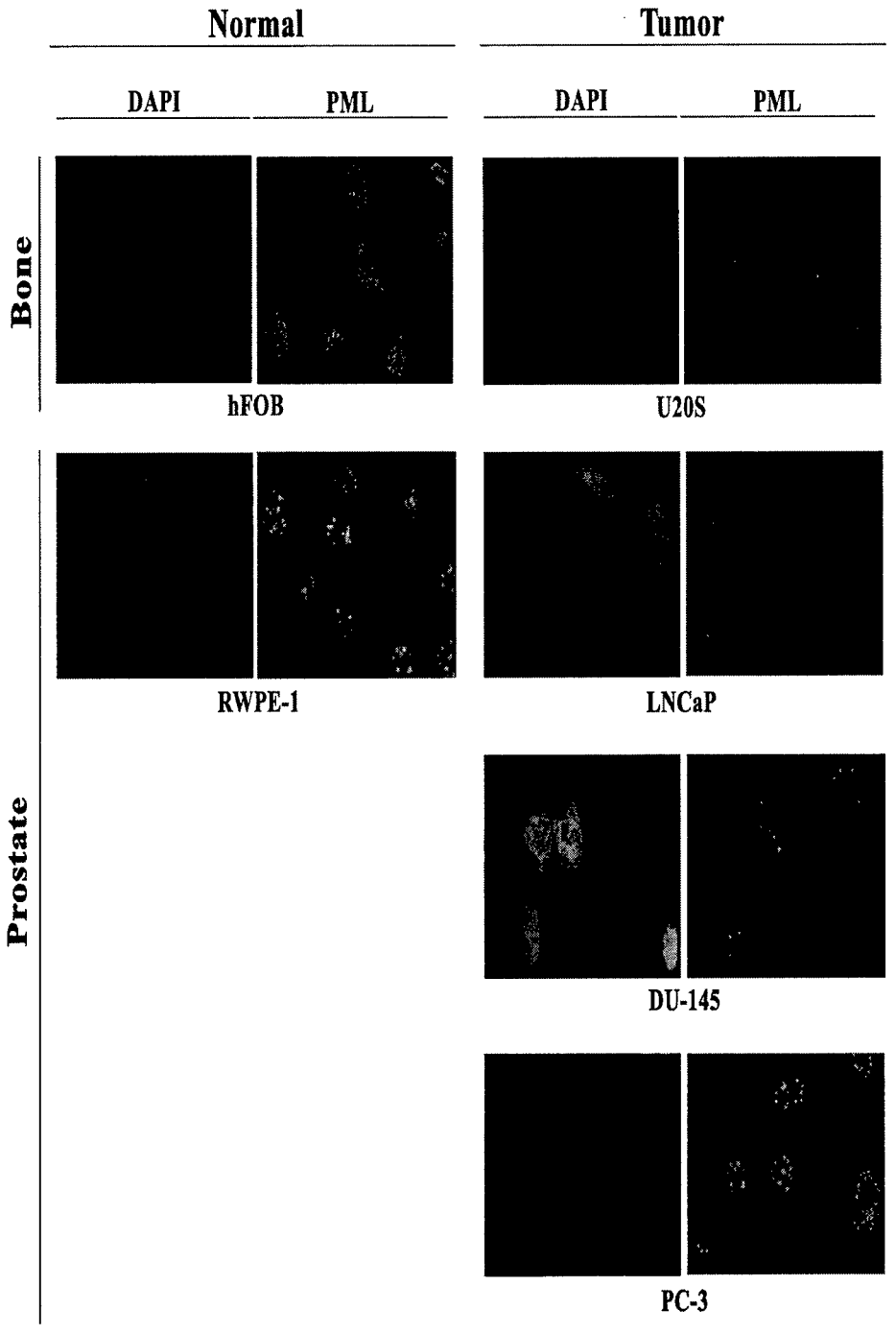

Figure 2. Immunofluorescent microscopy examining the expression and localization of the nuclear body protein PML. Monoclonal anti-PML antibody (red) detection of punctate PML in normal/tumor bone and prostate cell lines. Dapi (blue) identifies the nuclei.

Key Research Accomplishments

- Submission of a manuscript entitled "ICP0 prevents RNase L-independent rRNA cleavage in HSV-1-infected Cells" to Journal of Virology (accepted pending modification).

Reportable Outcomes

- Submission of a manuscript entitled "ICP0 prevents RNase L-independent rRNA cleavage in HSV-1-infected Cells" to Journal of Virology (accepted pending modification).

- Submission of grant application entitled "Herpes virus based oncolytic viruses for cancer therapy" to Canadian Institutes of Health Research, National Cancer Institute of Canada and The Ontario Cancer Research Network.

- Invitation to speak at the 2006 Gordon Research Conference on the Science of Viral Vectors, Gene Expression and Applications on our oncolytic virus program.

Conclusions

In conclusion, we have made progress on each of the four aims outlined in our original statement of work. In particular, the work outlined in the first aim was recently submitted for publication and accepted pending modification of the manuscript. In combination with our studies on breast cancer, the work supported by the US Army Medical Research and Materiel Command has yielded sufficient information to warrant submission of a full operating grant from the top two Canadian research institutes, CIHR and NCIC. We are grateful for the funds provided through the Exploration & Hypothesis Development program.

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ICP0 prevents RNase L-independent rRNA cleavage in HSV-1-infected Cells

Running Title: HSV-1 ICP0 blocks rRNA degradation

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ABSTRACT

The classical interferon (IFN)-dependent antiviral response to viral infection involves the regulation of IFN-stimulated genes (ISGs), one being the cellular endoribonuclease RNase L that arrests protein synthesis and induces apoptosis by nonspecifically cleaving ribosomal RNA (rRNA). Recently, the herpes simplex virus type-1 (HSV-1) protein ICP0 has been shown to block the induction of ISGs by subverting the IFN pathway upstream of the oligoadenylate synthetase (OAS)/RNase L pathway. We report that ICP0 also prevents rRNA degradation at late stages of HSV-1 infection, independent of its E3 ubiquitin ligase activity, and that the resultant rRNA degradation is independent of the classical RNase L antiviral pathway. Moreover, the degradation is independent of the viral ribonuclease vhs, and is independent of IFN response factor-3 (IRF-3). These studies indicate the existence of another, previously unidentified ribonuclease, that is part of the host antiviral response to viral infection.

INTRODUCTION

Viral infection of mammalian cells induces a robust antiviral response intended to restrict viral replication and propagation. A predominant characteristic of this innate immune response is the expression and secretion of interferons (IFNs), a family of immunomodulatory cytokines with antiviral and antiproliferative activities (43). Once secreted from infected cells, the type I IFNs (IFN α,β) bind to their cognate class II cytokine receptors in both paracrine and autocrine fashions, and induce the phosphorylation of Jak/Stat molecules, leading to the nuclear translocation of activated Stat1-Stat2-IRF9 heterotrimers. These complexes bind to the IFN-stimulated response element (ISRE) in promoters of IFN-stimulated genes (ISGs), resulting in the robust induction of these antiviral effector molecules. Among the ISGs most intently studied are the dsRNA-dependent protein kinase R (PKR), which induces protein synthesis arrest (52), and the ubiquitous 2'-5' oligoadenylate synthetase (OAS) family of proteins which function to promote RNA degradation (44). Once upregulated, PKR and OAS become activated by binding to dsRNA, a by-product of viral replication.

OAS catalyzes the synthesis of variable short 2'-5' linked oligoadenylates (2'-5'A) from ATP, which in turn activate the latent endoribonuclease RNase L to cleave cellular and viral RNA (36). Infection with several viruses, including vaccinia virus and encephalomyocarditis virus, leads to RNase L-dependent ribosomal RNA (rRNA) degradation (44). Ultimately, rRNA cleavage results in protein synthesis inhibition and apoptosis, and constitutes a significant cellular antiviral event to prevent viral propagation (7). Indeed, several viruses have evolved specific mechanisms to counteract the 2'-5'A pathway, including human immunodeficiency virus-1, vaccinia virus and the α-herpesvirus herpes simplex virus type 1 (HSV-1) (8, 27, 53). HSV-1 infection of conjunctival cells induces the synthesis of 2'-5'A derivatives, which

antagonize RNase L activation by competing with genuine 2'-5'A for binding to ankyrin repeats 7 and 8 on RNase L. In addition, HSV-1, like many other viruses, has been shown to inhibit IFN signaling pathways upstream of RNase L activation at multiple points (25, 31, 54, 55).

Taken together, it appears that preventing cellular rRNA degradation is of great importance to viral propagation, and viral interference in the IFN-regulated OAS/RNase L pathway is paramount for viral replication and spread. Despite these observations, there is conflicting evidence for the specific contribution of RNase L towards an antiviral state in HSV-1 infected cells. Studies with wild type (wt) and RNase L-knockout cells illustrated that HSV-1 infection does not significantly induce RNase L activity *in vitro* (47) and that the absence of RNase L does not significantly affect viral growth and virulence in an *in vivo* ocular model of HSV-1 infection (24). This observation is in contrast to another study that concluded HSV-1 infection of RNase L-knockout mice induces a significantly higher mortality rate and heightened susceptibility to herpetic disease and stromal keratitis compared to HSV-1 infection of wt infected mice (57). These data suggest that either RNase L does not significantly contribute to host defense against HSV-1 infection, or RNase L, as a component of the IFN signaling antiviral pathway, is inhibited during the course of HSV-1 infection by a viral factor.

Expressed early in infection, the HSV-1 immediate early (IE) protein infected cell protein 0 (ICP0) is a multifunctional transcriptional activator of viral and cellular genes that synergistically functions with another IE protein, ICP4, for several of its transcriptional functions (14). In the absence of ICP0, initiation of lytic replication is diminished, and latent genomes reactivate with decreased kinetics. In addition, ICP0 is responsible for surmounting a variety of cellular antiviral responses (14, 30-32). Upon translocating to the nucleus early in infection, ICP0 promotes the proteasome-dependent degradation of an array of cellular antiviral ISGs,

including the nuclear body-associated proteins promyelocytic leukemia protein (PML) and Sp100 (9, 15, 34). To date, ICP0's biological effects have been found to require the N-terminal RING finger domain, which mediates E3 ubiquitin ligase activity (3). The resultant disruption of ND10 nuclear bodies, in addition to other IFN-induced pathways, diminishes cellular antiviral capacity. Recently, ICP0 has been shown to block ISG expression by inhibiting the key transcriptional activators IFN regulatory factor (IRF) 3 and IRF7 (12, 25, 29). Moreover, ICP0 functions to counteract an IFN-induced barrier to virus replication (32, 33).

Since ICP0 is involved in subverting IFN signaling during the innate immune response to HSV-1 infection and RNase L-mediated rRNA degradation is a component of the cellular antiviral response, we set out to determine if ICP0 prevents cellular rRNA degradation during HSV-1 infection. We report that in the absence of ICP0 expression, HSV infection results in RNase L- and IRF3-independent rRNA degradation in a variety of cell types at late times post infection. The resultant rRNA degradation is independent of both the virion host shutoff (vhs) ribonuclease, and the E3 ubiquitin ligase activity of ICP0. These studies provide further evidence for the existence of another, previously unidentified cellular endoribonuclease that is part of a host antiviral response to viral infection.

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung (HEL) fibroblast, HepG2 hepatoma, U20S osteosarcoma and Vero monkey kidney epithelial cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (Vero) or 10% (HEL, HepG2 and U2OS) fetal bovine serum (FBS) and 2mM L-glutamine. RNase $L^{-/-}$ and RNase $L^{+/+}$ murine embryo fibroblasts (MEFs; genetic background C57BL/6) (58) and $IRF3^{-/-}$ and $IRF3^{+/+}$ MEFs (genetic background C57BL/6) (41) were maintained in α MEM supplemented with 10% FBS and 2mM L-glutamine. A listing of all HSV-1 viruses used in this study is provided in Table 1. AdMLP0 is a type 5 adenovirus expressing ICP0 under the major late promoter while AdE1E3 is a control adenovirus containing deletions of the E1 and E3 transcriptional units (59). Wild-type and IE mutant HSV-1 infections were completed at a multiplicity of infection (MOI) of 1 and 5 PFU per cell, respectively, in serum free DMEM for 1 hour. Adenovirus infections were completed at a MOI of 100 PFU/cell in phosphate buffered saline supplemented with 0.01% $MgCl_2$ and 0.01% $CaCl_2$ for 45 minutes. UV inactivation of viruses was performed with a UV Stratalinker 2400 (Stratagene) for the length of time required to drop infectious titres by greater than 5 orders of magnitude. Unless otherwise indicated, total cellular RNA was extracted 3 days after wt or mutant HSV infection, and 24 hours after adenovirus infection.

PolyIC Transfections and Treatments. Polyinosinic-polycytidylic acid (polyIC, Amersham-Pharmacia) was reconstituted in PBS at 5 mg/mL, denatured at 55°C for 15 min, and left to anneal at room temperature prior to transfection. Unless otherwise indicated, all cell lines were transfected with 1 μg polyIC per mL or 500 ng 2'-5'A (R. Silverman) using the Lipofectamine transfection reagent (Invitrogen) according to the manufacturers protocol.

RNA Extraction and Analysis. RNA was harvested using Trizol (Life Technologies) according to the manufacturer's recommendations. 5 μg of RNA was diluted into a 3-N-morpholino propansulfonic acid (MOPS) solution containing 20% formaldehyde and 50% formamide. Samples were subjected to agarose-gel electrophoresis (1% agarose, 20% formaldehyde) in MOPS running buffer in the presence of ethidium bromide (EtBr) for 1.5 hours at a potential difference of 100 V. RNA gels were scanned using the Typhoon Imager (Amersham Biosciences) using ImageQuant (Amersham Biosciences) software.

RESULTS

ICP0 Prevents HSV-1-induced rRNA Degradation. Given the role of ICP0 in mediating host antiviral responses, we sought to determine if ICP0 prevented rRNA degradation in HSV-1 infected cells by monitoring rRNA degradation following wt and ICP0-null HSV infection. Although ICP0 mutants are grown under complementing conditions, their titres are reproducibly 5-fold lower than that of wt virus (data not shown), due to an increase in the particle to pfu ratio (48). Thus in this and subsequent experiments, we utilized MOIs of 1 and 5 for wt and ICP0-null HSV infections, respectively. Under these conditions, the overall level and kinetics of viral protein expression in human embryonic lung (HEL) fibroblasts were similar (data not shown). As shown in Figure 1A, HEL fibroblasts infected with various ICP0-null HSV-1 mutants (described in Table 1), but not wt HSV-1, demonstrated cellular 28S and 18S rRNA degradation after 3 days of infection. To determine if the rRNA degradation pattern was consistent with an RNase L-mediated event, we transfected HEL fibroblasts with polyinosinic-polycytidylic acid (polyIC), a synthetic dsRNA polyribonucleotide derivative. rRNA degradation characteristic of RNase L activation was observed upon transfection of polyIC with Lipofectamine (LF), but not following treatment with either component on its own. Identical patterns were observed when 2'-5'A was used in place of polyIC (data not shown). The degradation patterns observed following transfection of polyIC or infection with ICP0-null HSV-1 were dissimilar, suggesting that RNase L may not mediate rRNA degradation following HSV-1 infection in the absence of ICP0 expression. One possible explanation for the disparate rRNA degradation profiles is a difference in degradation kinetics. To address this possibility, HEL fibroblasts were either transfected with polyIC or infected with ICP0-null HSV-1. As shown in Figure 1B, polyIC-induced rRNA degradation appears within the first hour of transfection, with complete 28S

rRNA degradation occurring within three hours, and involves the formation of a series of complex degradation products. In contrast, rRNA degradation following infection with ICP0-null HSV-1 is a late stage event, with observable degradation two days post-infection, and involves the formation of only two major rRNA degradation products.

In order to determine if the rRNA degradation following HSV-1 infection occurs in the absence of ICP0 with viral strains other than KOS, we infected HEL fibroblasts with wt strain 17 and its ICP0-null derivative dl1403. As shown in Figure 1C, dl1403 induced an identical degradation pattern to that of 7134, which was absent in $17syn^+$ infected cells. Infection with the strain 17-derived ICP0 RING finger domain mutant FXE, which has abolished E3 ubiquitin ligase activity, did not elicit rRNA degradation.

Previous studies of HSV-1 replication have shown that ICP0 functions synergistically with other IE proteins, such as ICP4, in mediating some of its biological functions (14). To determine if ICP0 is the sole IE protein required for the prevention of rRNA degradation, HEL fibroblasts were infected with a panel of single IE mutant viruses (refer to Table 1). Aside from 7134, no other IE mutant virus infection induced rRNA degradation (Figure 1D), illustrating that ICP0 is the only IE protein required for the prevention of rRNA degradation in HSV-1 infected HEL fibroblasts.

Cell type specificity of ICP0-null HSV-1-induced rRNA degradation. Since the expression of RNase L is cell type specific (21, 35), we set out to determine if the rRNA degradation observed in ICP0-null HSV-1 infected HEL fibroblasts can occur in other cell types. Infection of human osteosarcoma (U20S; Figure 2A) and hepatoma (HepG2; Figure 2B) cells with ICP0-null HSV-1 mutants induced an rRNA degradation pattern similar to that in HEL fibroblasts, while KOS-infected cells displayed intact rRNA. Infection of green monkey kidney epithelial (Vero)

and human alveolar epithelial (A549) cells with ICP0-null HSV-1 induced rRNA degradation comparable to that of U20S cells (data not shown). Notably, the extent of rRNA degradation was reproducibly less in these cell lines as compared to HEL fibroblasts (Figure 1A). As with HEL fibroblasts, polyIC transfection induced rRNA degradation in A549, Vero and U20S cells, indicating the presence of functional RNase L. In contrast, polyIC transfection of HepG2 cells did not induce rRNA degradation (Figure 2B), which is consistent with the lack of functional RNase L in this cell line (49).

rRNA degradation in ICP0-null HSV-1 infected cells is independent of RNase L. Given the difference in rRNA degradation patterns between transfected and infected cells (Figure 1A and B) and the ability to induce rRNA degradation following infection in RNase L-deficient HepG2 cells (Figure 2B), we set to conclusively determine the role of RNase L in mediating rRNA degradation following ICP0-null HSV-1 infection. We infected wt murine embryo fibroblasts (MEFs; Figure 3A) and RNase $L^{-/-}$ MEFs (Figure 3B) with KOS, 7134, dlX3.1, or n212. Both wt and RNase $L^{-/-}$ MEFs infected with ICP0-null HSV-1 displayed an rRNA degradation pattern similar to that in HEL fibroblasts. Consistent with the lack of RNase L, polyIC-induced rRNA degradation was observed in wt but not RNase $L^{-/-}$ MEFs. Taken together, the rRNA degradation observed following HSV-1 infection in the absence of ICP0 expression occurs independent of RNase L.

rRNA degradation in ICP0-null HSV-1 infected cells is independent of the viral ribonuclease vhs. Since the rRNA degradation observed following ICP0-null HSV-1 infection occurs in the absence of RNase L, then either a viral or an alternative cellular ribonuclease is responsible for the observed degradation. Previously, the HSV-1 virion host shutoff protein

(vhs) was shown to induce a rapid arrest of macromolecular biosynthesis by associating with the cellular transcription factor eIF4H and inducing non-specific destabilization of cellular and viral mRNA (45). Due to its nuclease activity, we sought to determine if vhs contributes to the rRNA degradation observed following HSV-1 infection in the absence of ICP0 expression by infecting HEL fibroblasts with wt HSV-1, or mutants deficient in ICP0 and/or vhs. While ICP0-null HSV (7134 and 7134Δsma) infected cells displayed rRNA degradation, KOS and vhs-null HSV infected cells possessed intact rRNA (Figure 4), illustrating that the rRNA degradation observed following HSV-1 infection in the absence of ICP0 expression is independent of vhs.

ICP0 overexpression does not prevent RNase L-mediated rRNA degradation. We have shown that infection with HSV-1 leads to RNase L-independent rRNA degradation that is blocked upon expression of ICP0. However, it remains unclear if ICP0 is capable of also counteracting the IFN-mediated RNase L pathway. To determine if ICP0 expression is sufficient to block RNase L-mediated rRNA degradation, we infected Vero cells with an adenovirus encoding ICP0 under the transcriptional control of the major late promoter (AdMLP0), and subsequently challenged the infected cell cultures with polyIC transfection or ICP0-null HSV infection (7134). Immunofluorescence microscopy illustrated that approximately 80% of cells expressed ICP0 following AdMLP0 infection (data not shown). In addition, western blot analysis of AdMLP0- and KOS-infected cell lysates indicated that both viruses expressed similar amounts of ICP0 (data not shown). While ICP0 pre-expression prevented rRNA degradation in 7134-infected cells, thus restoring a wt phenotype, it did not prevent polyIC-induced, RNase L-mediated rRNA degradation (Figure 5), illustrating that ICP0 does not inhibit RNase L-mediated rRNA degradation. Infection with a control adenovirus (AdE1E3) did not complement the ICP0-null phenotype. Moreover, polyIC transfection following KOS infection induced rRNA

degradation, indicating that wt HSV does not prevent RNase L-mediated rRNA degradation (data not shown). Similar results were observed in AdMLP0-infected A549 cells (data not shown). To corroborate these observations, we utilized 0-28 cells, a stable Vero-derived cell line that expresses ICP0 under the control of its endogenous promoter. Under these conditions, similar results to Figure 5 were observed (data not shown). Therefore, whether expressed by a recombinant adenovirus, wt HSV or within a stable cell line, ICP0 expression does not prevent RNase L-mediated rRNA degradation.

rRNA degradation in ICP0-null HSV-1 infected cells is independent of IRF3. Given that rRNA degradation is a hallmark of apoptosis (19), and that ICP0 blocks IRF3 (25), a transcription factor implicated in apoptosis (20), we set out to determine the role of IRF-3 in RNase L-dependent and -independent rRNA degradation. Wt and $IRF3^{-/-}$ MEFs (Figure 6A and 6B, respectively) were transfected with polyIC or infected with wt or ICP0-null HSV-1. Both wt and $IRF3^{-/-}$ MEFs infected with ICP0-null HSV-1 displayed rRNA degradation similar to that observed in HEL fibroblasts. Notably, polyIC-mediated rRNA degradation was not observed in $IRF3^{-/-}$ MEFs, suggesting that IRF3 is essential for RNase L-mediated rRNA degradation.

To confirm these results, we investigated rRNA profiles following transfection of polyIC or infection with ICP0-null HSV-1 in Jak1-deficient parental cells (U4C) or a derivative, P2.1, that expresses only low levels of IRF3 (23). While polyIC-induced, RNase L-mediated rRNA degradation was markedly reduced in P2.1 cells compared toU4C cells, HSV-1-mediated rRNA degradation was similar in both (data not shown). Taken together, these data indicate that while ICP0-null HSV-1-induced rRNA degradation is IRF3-independent, polyIC-induced, RNase L-mediated rRNA degradation requires IRF3.

DISCUSSION

The innate antiviral response to viral replication involves the combined activities of ISGs in an effort to suppress viral replication and to induce apoptosis of infected cells to limit viral spread. Once activated by dsRNA, the endoribonuclease RNase L mediates both of these responses following infection with viruses such as vaccinia virus and encephalomyocarditis virus (7). Although HSV-1 has been shown to block RNase L-mediated RNA degradation by synthesizing decoy 2-5A derivatives that antagonize RNase L activity (8), the specific contribution of RNase L towards host antiviral capacity remains controversial. However, HSV-1 countermeasures to other IFN pathways are well documented (30).

Since the HSV-1 IE protein ICP0 has been shown to be instrumental in surmounting several of these IFN-dependent antiviral pathways, we investigated the possibility that ICP0 prevents cellular rRNA degradation following HSV-1 infection by blocking RNase L activity. We observed that in the absence of ICP0 expression, HSV-1 strains 17 and KOS induced rRNA degradation at late stages post-infection. Individual deletion of the remaining IE gene products failed to demonstrate hallmarks of rRNA degradation. While this observation suggests that ICP0 is necessary to block rRNA degradation, it does not discount the possibility that an additional viral protein(s) is also involved. It is well established by Blaho and colleagues that ICP27-null HSV infection results in many of the hallmarks associated with apoptosis through the destabilization of cellular Bcl-2 protein and a reduction in Bcl-2 RNA levels, including DNA fragmentation (1, 56). However, evidence exists which illustrates that rRNA degradation is a cell-type dependent occurrence that is independent of DNA fragmentation (39).

Intracellular antiviral pathways are predominantly mediated by immediate early response factors and are thus activated at early times of infection. Here, however, we detected rRNA

degradation at late times of infection. While we currently do not fully understand the biological implications of a delayed cellular immune response, a number of factors could impact on the delayed kinetics we observed. As mentioned above, it is likely that viral proteins other than ICP0 assist in blocking this cellular antiviral response. Viruses routinely encode multiple proteins to disable cellular antiviral pathways (40, 42). Under conditions where only ICP0 activity is absent, rRNA degradation would remain partially inhibited with subsequent degradation products requiring sufficient accumulation to become visible. Furthermore, these experiments were performed *in vitro* using relatively high multiplicities, conditions that may not reflect *in vivo* infections. We believe that these events are relevant to HSV biology, however, since rRNA degradation in the absence of ICP0 was observed in all cell types tested, particularly in mouse embryo and HEL fibroblasts. As opposed to the continuous cell lines tested, these fibroblasts are non-immortalized and non-transformed. Given that IFN is both anti-viral and anti-proliferative in nature, it is likely that IFN-mediated immune responses are more intact in "primary" cells as opposed to immortalized or transformed cells. Furthermore, in a series of burst experiments, we noted a decrease in ICP0-null viral titres that correlated with an increase in rRNA degradation, whereas no significant drop in wt viral titres was observed during the course of the experiment (data not shown).

Of particular interest, the ICP0 RING finger mutant was capable of blocking rRNA degradation, illustrating that the E3 ubiquitin ligase activity of ICP0 does not contribute to preventing rRNA degradation. Although ICP0 contains multiple functional domains, including a nuclear localization signal (NLS), a herpesvirus associated ubiquitin specific protease (HAUSP) binding domain and an ND10 localization domain, the biological functions of ICP0 have to date been found to rely on its E3 ubiquitin ligase activity (14, 25). Therefore, this is the first ICP0-

regulated biological phenomenon that is independent of ICP0's E3 ubiquitin ligase activity. Studies are underway to determine the mechanism(s) whereby ICP0 blocks rRNA degradation following HSV-1 infection.

There are three mutually exclusive mechanisms that could account for the observed rRNA degradation following ICP0-null HSV-1 infection. In the first mechanism, RNase L mediates cellular rRNA degradation in response to HSV-1 infection, and ICP0 prevents this by either directly inhibiting RNase L or indirectly blocking an upstream activator of the OAS-RNase L pathway. We provide evidence, however, that RNase L is not mediating rRNA cleavage following HSV-1 infection. In addition to the RNase L-deficient hepatoma cell line (HepG2), both control MEFs and RNase L knockout MEFs infected with ICP0-null HSV-1 displayed rRNA degradation. Furthermore, RNase L-mediated rRNA degradation exhibits markedly different kinetics from that of ICP0-null HSV-1-induced rRNA degradation and produces a disparate rRNA degradation profile. In addition, ICP0 overexpression did not prevent RNase L specific rRNA cleavage, further illustrating that ICP0 does not block the OAS-RNase L pathway during HSV-1 infection. Recently, a polyIC-containing liposome complex (NS-9) was shown to induce rRNA degradation in an IRF3-dependent manner (51). In agreement with these data, we report that polyIC-induced, RNase L-mediated rRNA degradation is IRF3 dependent, and further conclude that ICP0-null HSV-1-induced rRNA degradation is IRF3-independent. Taken together, these data illustrate that ICP0 prevents an RNase L- and IRF3-independent rRNA degradation event that is induced following HSV-1 infection. These results also parallel those of other studies that determined RNase L activity does not contribute to the host antiviral response during HSV-1 infection (24, 47). Interestingly, RNase L activity does

not contribute to cellular antiviral responses during infection with VZV, a related α-herpesvirus (11).

In a second putative mechanism, the rRNA degradation observed following ICP0-null HSV-1 infection is mediated by the viral ribonuclease vhs. However, in the absence of ICP0 and vhs, rRNA degradation was still prominent in HEL fibroblasts, discrediting vhs as the causative ribonuclease. This conclusion is in agreement with previous research illustrating that rRNA is resistant to vhs-mediated degradation (16, 22).

In the last mechanism, HSV-1 induces the activity of a cellular ribonuclease other than RNase L, and ICP0 blocks the resultant rRNA degradation. Indeed, certain cytopathic strains of hepatitis A and the murine coronavirus mouse hepatitis virus have been shown to induce rRNA degradation independent of RNase L and of other known viral and cellular ribonucleases (2, 18). Several lines of research are currently underway to determine the identity of this causative ribonuclease, its effects on cell viability and apoptosis, and the biological significance of rRNA degradation induced by ICP0-null HSV-1 infection. In conclusion, ICP0-null HSV-1 infection induces cellular rRNA degradation in a variety of cell types that is independent of the classical RNase L pathway. Furthermore, the resultant degradation is independent of the viral ribonuclease vhs, and ICP0 prevents this cellular response to infection in a manner independent of its E3 ubiquitin ligase activity. Although the specific mechanism of this rRNA degradation remains unknown, these studies indicate the existence of another ICP0-mediated viral countermeasure to the antiviral response against HSV-1 infection, and provide evidence for the existence of a previously unidentified ribonuclease that is part of the host antiviral response to viral infection.

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FIGURE LEGENDS

Figure 1. ICP0 prevents rRNA degradation induced following infection of HEL fibroblasts with HSV-1. RNA gel electrophoresis was performed in fibroblasts treated with Lipofectamine (LF), polyIC or LF+polyIC, or infected with wild-type and mutant HSV-1 viruses of strain KOS (A, B and D) or 17 (C).

Figure 2. The extent of rRNA degradation observed following ICP0-null HSV-1 infection is cell type specific. RNA gel analysis was performed on U20S (A) and HepG2 (B) cells transfected with polyIC or infected with the indicated HSVs (strain KOS).

Figure 3. The rRNA degradation observed following ICP0-null HSV-1 infection is independent of RNase L. RNA gel analysis of wt MEFs (A) or RNase $L^{-/-}$ MEFs (B) transfected with polyIC or infected with wt HSV-1 or ICP0-null HSV-1 (strain KOS).

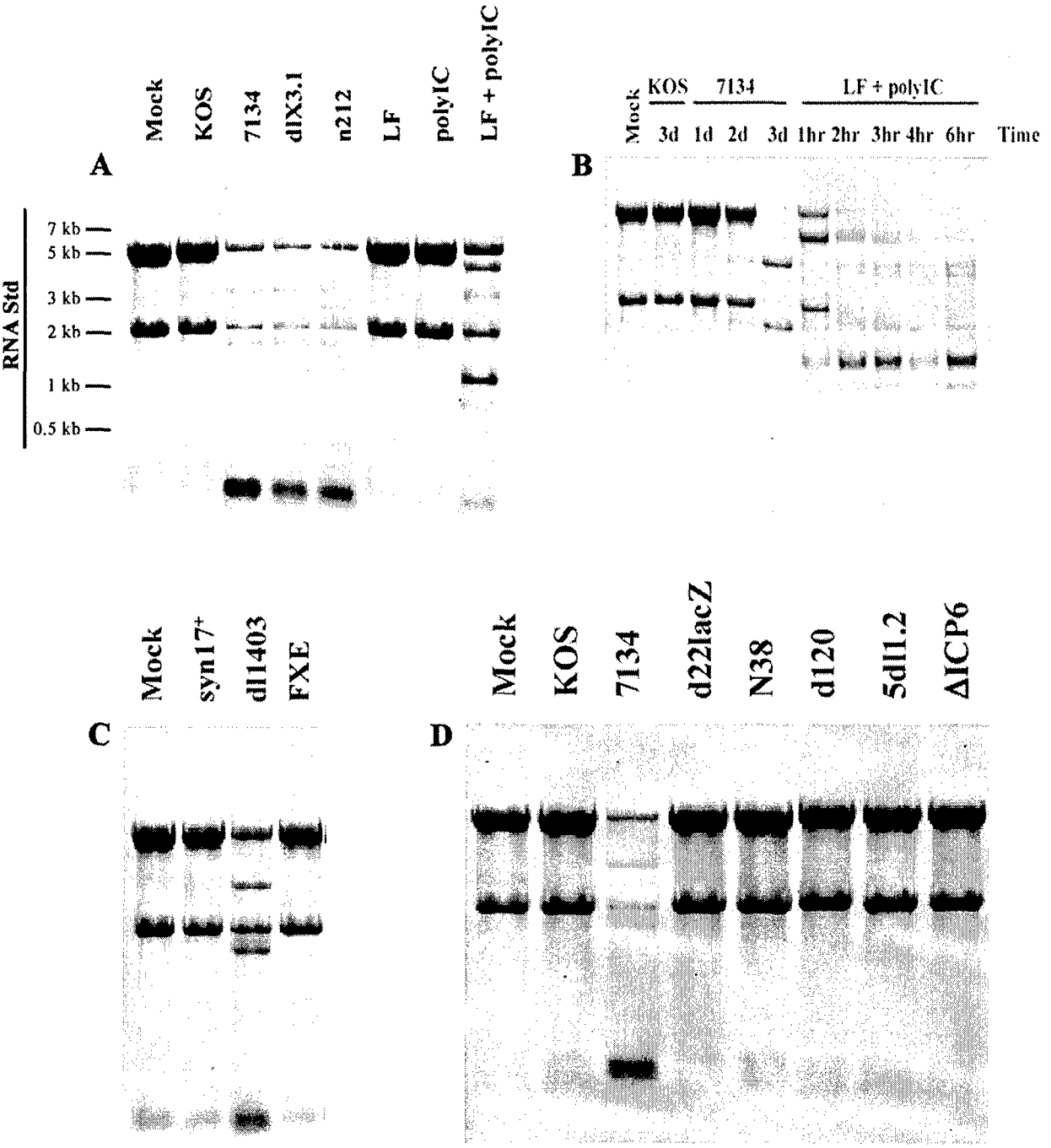
Figure 4. The rRNA degradation observed following ICP0-null HSV-1 infection is independent of the viral ribonuclease vhs. RNA gel analysis of HEL fibroblasts infected with the indicated ICP0-null, vhs-null, or ICP0/vhs-null HSV-1 mutant viruses (strain KOS).

Figure 5. ICP0 overexpression does not prevent RNase L-specific rRNA degradation. Vero cells were infected with control adenovirus (AdE1E3) or adenovirus encoding ICP0 (AdMLP0), and subsequently challenged with polyIC transfection (10ng), or HSV-1 infection.

Figure 6. The rRNA degradation observed following ICP0-null HSV-1 infection is independent of IRF3. RNA gel analysis was performed on wt MEFS (A) or $IRF3^{-/-}$ MEFs (B) transfected with polyIC or infected with the indicated wt and ICP0-null mutant HSVs (strain KOS).

Table 1. Properties of HSV-1 viruses used in this study.

Virus	Strain	Mutation	Reference
KOS	KOS	Wild type	(46)
7134	KOS	ICP0-null	(6)
dlX3.1	KOS	ICP0-null	(38)
n212	KOS	ICP0-null	(5)
Δsma	KOS	vhs-null	(37)
7134Δsma	KOS	ICP0/vhs-null	(25)
$17syn^+$	17	Wild-type	(4)
dl1403	17	ICP0-null	(48)
FXE	17	RING finger deletion	(13)
d22lacZ	KOS	ICP22-null	(26)
N38	KOS	ICP47-null	(50)
ΔICP6	KOS	ICP6-null	(17)
d120	KOS	ICP4-null	(10)
5dl1.2	KOS	ICP27-null	(28)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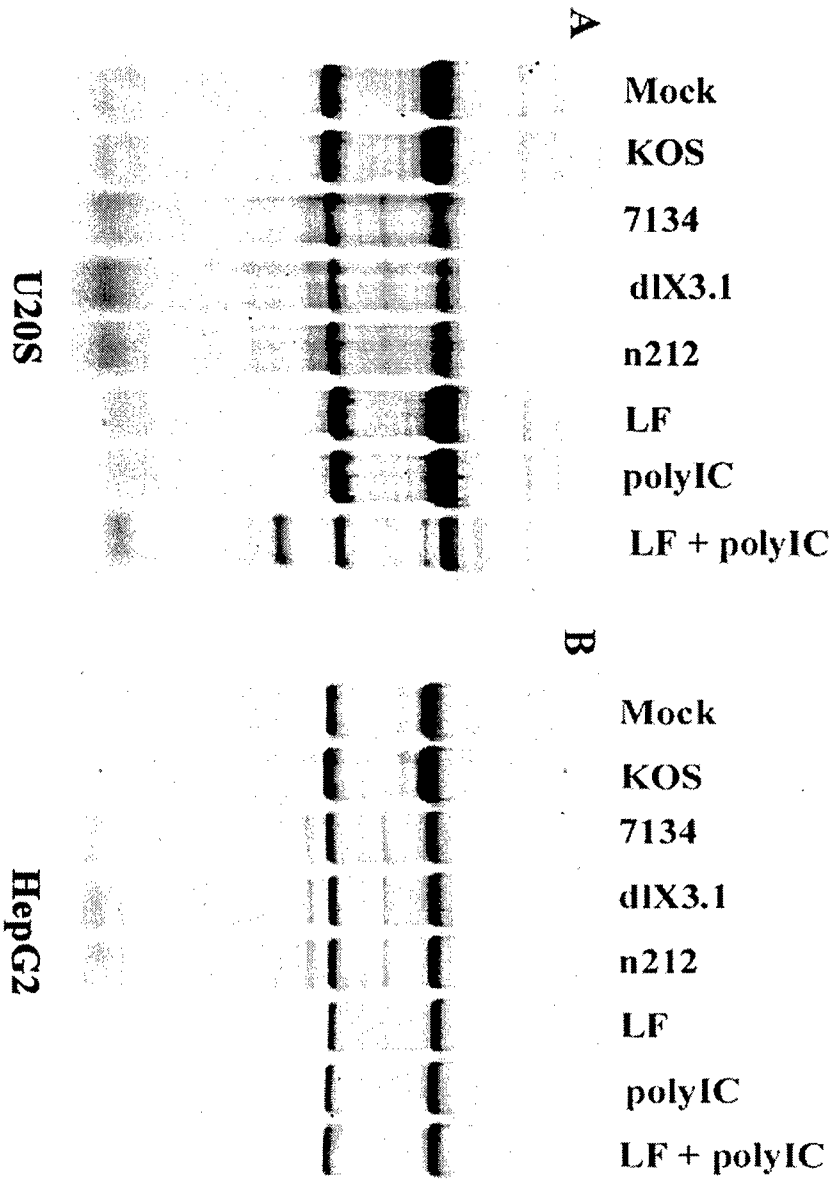
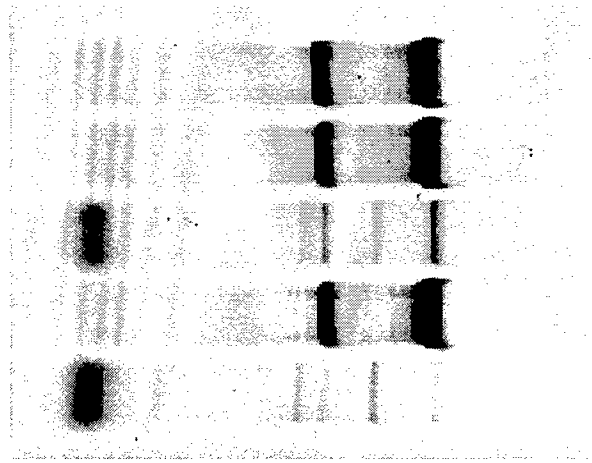
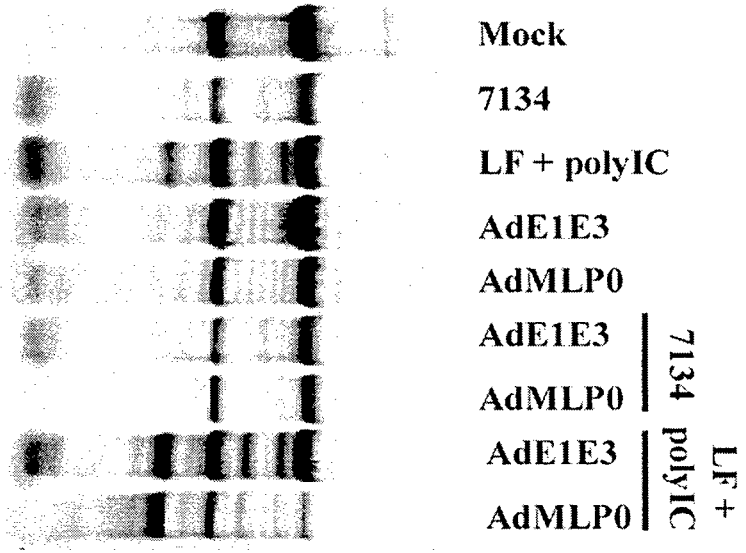
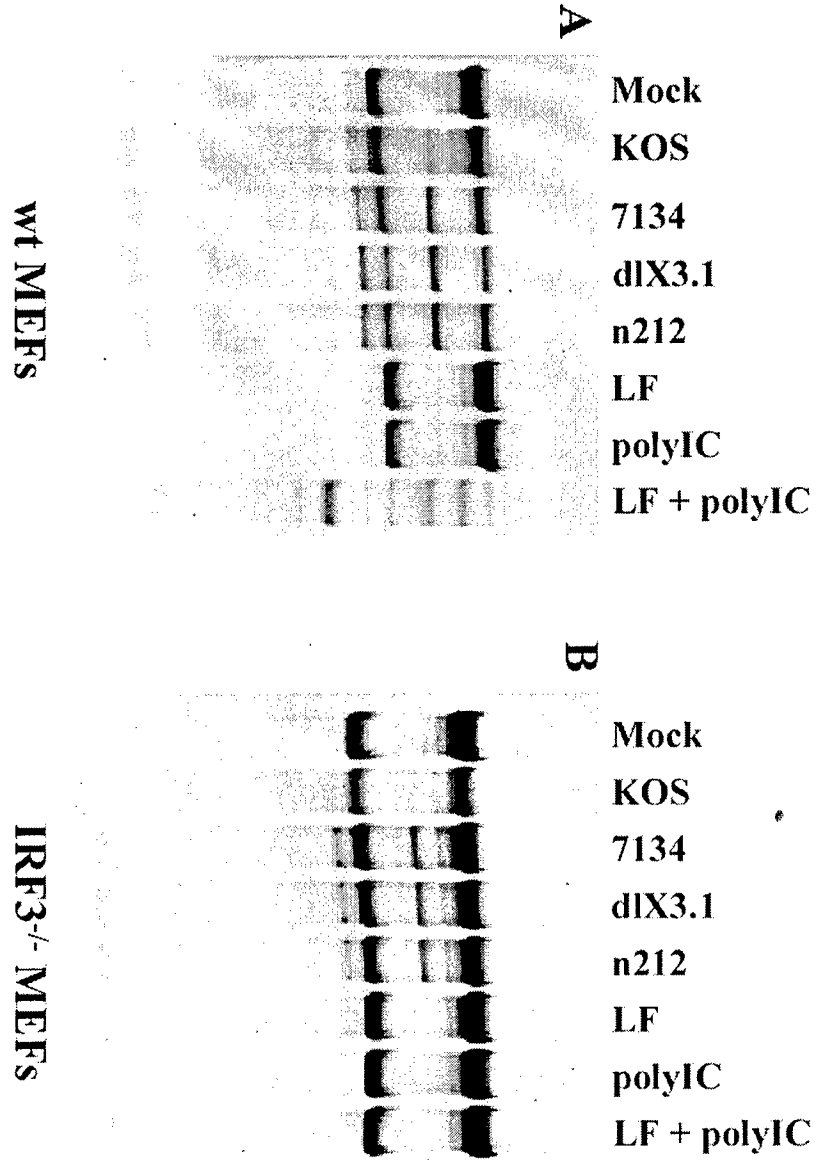
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC
RNA Std
7 kb
5 kb
3 kb
2 kb
1 kb
0.5 kb
B
Mock
KOS
7134
LF + polyIC
3d 1d 2d 3d 1hr 2hr 3hr 4hr 6hr
Time
C
Mock
syn17+
dl1403
FXE
D
Mock
KOS
7134
d22lacZ
N38
d120
5dl1.2
ΔICP6


A
U20S
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC
B
HepG2
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC

A
wt MEFs
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC
B
RNase L-/- MEFs
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC


Mock
KOS
7134
Δsma
7134Δsma


Mock
7134
LF + polyIC
AdE1E3
AdMLP0
AdE1E3
AdMLP0
7134
AdE1E3
AdMLP0
LF + polyIC


A
wt MEFs
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC
B
IRF3-/- MEFs
Mock
KOS
7134
dlX3.1
n212
LF
polyIC
LF + polyIC