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Analysis of the cell cycle regulatory protein (E2F1) after infection of cultured cells with bovine herpesvirus 1 (BHV-1) or herpes simplex virus type 1 (HSV-1)

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SUMMARY

The E2F family of cellular transcription factors controls cell cycle progression and cell death. During cell cycle progression, activated cyclin-dependent kinases phosphorylate the retinoblastoma (Rb) protein, causing the release and activation of E2F family members. Previous studies demonstrated that bovine herpes virus 1 (BHV-1) productive infection increases E2F1 protein levels, the bICP0 early promoter is activated more than 100 fold by E2F1 or E2F2, and silencing E2F1 reduced the efficiency of productive infection. In this study, the effect of HSV-1 productive infection on E2F protein levels and regulation of E2F dependent transcription was compared to BHV-1 infection in the same permissive cell line, rabbit skin (RS) cells. Silencing E2F1 with a specific siRNA reduced herpes simplex virus type 1 (HSV-1) productive infection approximately 10 fold in RS cells, and total E2F1 protein levels increased during productive infection. In contrast to RS cells infected with BHV-1, a fraction of total E2F1 protein levels was localized to the cytoplasm in HSV-1 infected RS cells. Furthermore, E2F1 did not efficiently trans-activate the HSV-1 ICP0 or ICP4 promoter. When RS cells were transfected with an E2F reporter construct or the cyclin D1 promoter and then infected with BHV-1, promoter activity increased after infection. In contrast, HSV-1 infection of RS cells had little effect on E2F dependent transcription and cyclin D1 promoter activity was reduced. In summary, these studies indicated that silencing E2F1 reduced the efficiency of HSV-1 and BHV-1 productive infection. However, only BHV-1 productive infection induced E2F dependent transcription.

Keywords

herpes simplex virus type 1; bovine herpesvirus 1; E2F dependent transcription; productive infection

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INTRODUCTION

During productive infection of cultured cells, gene expression of herpes simplex virus type 1 (HSV-1) and bovine herpesvirus 1 (BHV-1), both alpha-herpesvirinae subfamily members, is temporally regulated in three distinct phases: immediate early (IE), early (E), or late (L), reviewed in (Jones, 1998; 2003). In contrast to small DNA tumor viruses, alpha-herpesvirinae subfamily members do not appear to promote entry into the S phase of the cell cycle. For example, HSV-1 encodes several genes, ICP0, ICP27, ICP22/US1.5, and UL13, which inhibit cell cycle progression (Advani *et al.*, 2000; Flemington, 2001; Hobbs & DeLuca, 1999; Lomonte and Everett, 1999; Orlando *et al.*, 2006; Song *et al.*, 2001). However, drugs that interfere with cyclin dependent kinase activity, roscovitine and olomucine, reduce the efficiency of productive infection (Schang *et al.*, 1999; Schang *et al.*, 1998) suggesting that certain cell cycle regulatory proteins facilitate HSV-1 replication. The E2F family of transcription factors is cell cycle regulatory proteins that interact with Rb family members (Rb, p107, and p130) (Harbour & Dean, 2000). Phosphorylation of Rb family members by cyclin dependent kinase/cyclin complexes leads to E2F release, and consequently certain E2F family members (E2F1, -2, or -3) activate transcription (Attwooll *et al.*, 2004; Harbour & Dean, 2000; Nevins *et al.*, 1997; Weintraub *et al.*, 1992). Consensus E2F binding sites are present in the promoters of many genes that control cell cycle progression (DeGregori *et al.*, 1995; Nevins *et al.*, 1997; Ohtani *et al.*, 1995; Schulze *et al.*, 1995; Wells *et al.*, 1997). Many DNA synthetic genes are activated by E2F family members (Harbour & Dean, 2000) suggesting transient induction of E2F dependent transcription by alpha-herpesvirinae subfamily members may enhance productive infection in highly differentiated cells.

The E2F family of transcription factors stimulates BHV-1 productive infection and reactivation from latency. For example, a recent study demonstrated that a siRNA directed against E2F1 inhibits BHV-1 productive infection, E2F1 protein levels and binding activity increase after infection, and E2F1 or E2F2 stimulates the bICP0 early promoter more than 100 fold (Workman and Jones, 2010). Furthermore, over-expression of E2F4 stimulates BHV-1 productive infection and E2F1 or E2F2 trans-activates IEt1 (immediate early transcription unit 1) promoter activity (Geiser and Jones, 2003). During dexamethasone-induced reactivation from BHV-1 latency, sensory neurons that express abundant levels of lytic cycle genes also express cyclin E and cyclin A (Winkler *et al.*, 2000). Although these studies suggest that increased E2F protein levels and binding activity are important for productive infection, BHV-1 may also increase E2F1 protein levels because productive infection leads to p53 dependent apoptosis (Devireddy & Jones, 1999).

With respect to HSV-1 productive infection, “free” E2F (not associated with Rb family members) increases following infection of a human tumor cell line (C33-A) (Hilton *et al.*, 1995). Re-localization of E2F4 to the nucleus occurs in human tumor cell lines (C33-A and U2-OS) following HSV-1 infection (Olgiate *et al.*, 1999). Further support for E2F4 playing a role in HSV-1 replication comes from the findings that infection of mouse cells that lack p107^{-/-} and p130^{-/-}, two Rb family members that interact with E2F4, leads to reduced infectious virus (Ehmann *et al.*, 2001). In primary human fibroblasts or Hela cells, the subcellular distribution of E2F4 is altered following HSV-1 infection, which is assumed to inactivate E2F-4 (Advani *et al.*, 2000). This same study also concluded that HSV-1 infection leads to post-translational modification of E2F1 and E2F5, translocation of E2F family members from the nucleus to the cytoplasm, and reduced E2F binding to consensus E2F binding sites. Based on these observations, HSV-1 appears to inactivate E2F dependent signaling.

In this study, we compared the effects of HSV-1 versus BHV-1 infection on E2F1 and E2F dependent transcription in the same cell line. Like BHV-1, an E2F1-specific siRNA reduced HSV-1 productive infection following infection of rabbit skin cells. Although BHV-1 and HSV-1 both increased E2F1 protein levels, a significant amount of the E2F1 protein was localized in a cytoplasmic fraction following HSV-1 infection. Conversely, E2F1 was localized to the nucleus following BHV-1 infection. BHV-1, but not HSV-1, infection of rabbit skin cells enhanced E2F dependent transcription.

RESULTS

An E2F1 specific siRNA reduced the levels of HSV-1 productive infection

Silencing E2F1 decreases the levels of BHV-1 productive infection, in part, because E2F1 stimulates the bICP0 early (E) promoter more than 100 fold in transient transfection assays (Workman and Jones, 2010). Since BHV-1 and HSV-1 are both alpha-herpesvirinae subfamily members, we hypothesized that both viruses require similar transcription factors, including E2F1, for efficient productive infection. To test whether E2F1 influenced HSV-1 productive infection, we examined the effect of an E2F1 specific siRNA on viral growth in rabbit skin (RS) cells, which supports productive infection by BHV-1 and HSV-1. RS cells are not transformed by a known oncogene, they can be readily transfected with siRNA and plasmids (Workman and Jones, 2010), and thus are well suited for these studies. Relative to the control siRNA, co-transfection of the E2F1 siRNA, but not the control siRNA, with the HSV-1 genome reduced the number of plaques more than 10-fold (Figure 1A).

Previous studies demonstrated that the E2F1 specific siRNA reduced E2F1 protein levels, but not E2F2 or β -actin protein levels (Workman and Jones, 2010). Additional studies were performed to evaluate the effect of the E2F1 siRNA on the cell cycle in uninfected cells. The E2F1 siRNA reduced the percent of cells in S phase less than 2 fold at 24 hours after transfection (Figure 1B). However, silencing of E2F1 did not dramatically increase apoptosis or cell death. By 48 hours after transfection, there was no difference in the percent of cells in S phase regardless of the treatment. In summary, these studies demonstrated that silencing E2F protein levels reduced HSV-1 productive infection, which was similar to the effect of the same siRNA on BHV-1 productive infection (Workman and Jones, 2010).

Analysis of E2F1 trans-activation of HSV-1 promoter constructs

Previous studies revealed that E2F1 trans-activated the BHV-1 bICP0 early (E) promoter more than 100 fold in transient transfection assays (Workman and Jones, 2010). Two E2F1 responsive regions (ERR) were identified within the bICP0 early promoter, one adjacent to the TATA box (ERR1) and one approximately 600 bp upstream of the TATA box (ERR2). When cloned upstream of a minimal HSV-1 thymidine kinase promoter, ERR1 stimulated promoter activity 6 fold whereas ERR2 stimulate promoter activity more than 20 fold. Mobility shift assays provided further evidence that E2F1 interacted directly with these sequences (Workman and Jones, 2010). Therefore, we hypothesized that E2F1 may stimulate promoters of important HSV-1 regulatory genes.

To test this hypothesis, neuro-2A cells were cotransfected with an HSV-1 ICP0 or ICP4 promoter construct plus an E2F1 expression plasmid, and CAT activity was measured. Neuro-2A cells were used for this study because these cells are neuronal-like and it was of interest to test whether E2F regulates gene expression in neurons. Furthermore, E2F1 over-expression is not as toxic in neuro-2A cells when compared to RS cells (data not shown). The HSV-1 ICP0 promoter was activated only 3 fold by E2F1 (Figure 2A). The ICP4 promoter was consistently lower than basal activity when cotransfected with E2F1 (Figure 2A).

We may have under-estimated the effects of E2F1 on ICP0 and ICP4 promoter activity because E2F family members can induce apoptosis (Attwooll *et al.*, 2004; Chang *et al.*, 2000; Harbour & Dean, 2000) and the HSV-1 ICP0 as well as the ICP4 construct had high basal activities. Consequently, we examined whether reducing E2F1 protein levels by the E2F1 specific siRNA had an effect on ICP0 and ICP4 basal promoter activity. Neuro-2A cells were cotransfected with the designated promoter-CAT construct and the E2F1 siRNA or a control siRNA (cont-si). At 48 hours after transfection, cells were collected and CAT activity measured. The E2F1 specific siRNA reduced ICP0 promoter activity approximately 60%: whereas the control siRNA did not reduce ICP0 promoter activity (Figure 2B). The E2F1 siRNA reduced ICP4 promoter activity by approximately 40%. In contrast, the control siRNA reduced ICP4 promoter activity less than 20% (Figure 2B).

Finally, we tested whether E2F1 had an effect on a panel of HSV-1 promoter-luciferase constructs. Of those examined, the early UL42 promoter was activated approximately 2.5 fold (Figure 2C). UL42 encodes a protein that stimulates the viral DNA polymerase (Gottlieb & Challberg, 1994). Since E2F1 activates expression of genes that are necessary for DNA replication (DeGregori *et al.*, 1995), this result suggested that under certain conditions E2F1 might promote expression of certain viral genes that are necessary for DNA replication. The late VP16 and gC promoters as well as the early ICP6 promoter were not activated by E2F1. In summary, the HSV-1 promoters we examined were not efficiently trans-activated by E2F1 in neuro-2A cells.

Analysis of E2F1 protein levels during productive infection

Previous studies demonstrated that E2F1 protein levels increased during BHV-1 productive infection in bovine kidney cells (Workman and Jones, 2010). Additional studies were performed to directly compare the effect of BHV-1 or HSV-1 productive infection on E2F1 protein levels in RS cells. RS cells were infected with BHV-1 or HSV-1 using a moi of 5 and whole cell lysate collected at the designated times post-infection. Western blot analysis revealed that BHV-1 increased E2F1 protein levels by 16 hours after infection {(Workman and Jones, 2010) and (Figure 3A)}. Higher levels of E2F1 were detected in RS cell within 8 hours after infection with HSV-1 using a moi of 5 (Figure 3B). When RS cells were infected with HSV-1 at a moi of 0.5 (Figure 3C) or 0.1 (data not shown), enhanced E2F1 levels were not readily detected until 16 hours after infection. In RS cells, HSV-1 grows faster than BHV-1 at a moi of 0.5 or lower; however, the end point titers of BHV-1 and HSV-1 are similar when RS cells are infected with a moi of 0.5 or higher (data not shown). We suggest that differences in the growth rate of the two viruses was one reason why higher levels of E2F1 were detected in HSV-1 infected RS cells at 8 hours after infection when RS cells were infected with a moi of 5.

The E2F1 band detected after HSV-1 infection appeared to migrate differently compared to E2F1 after BHV-1 infection or in mock-infected cells (Figure 4A). E2F can be phosphorylated and phosphorylation is crucial for regulating transcriptional activation, localization, and stability (Garcia-Alvarez *et al.*, 2007; Real *et al.*, 2010; Weei-Chin *et al.*, 2011). To test whether E2F1 was phosphorylated, whole cell extract from HSV-1 infected cells was treated with calf intestinal alkaline phosphatase (CIAP) and then western blot analysis performed using an anti-E2F1 antibody (Figure 4B). CIAP treatment resulted in a decrease in the electrophoretic mobility of E2F1 in RS cells (Figure 4B) suggesting the observed shift in E2F1 mobility after HSV-1 infection was the result of phosphorylation. To visualize E2F1 in mock-infected cells, 200 ug of protein in the cell lysate was loaded versus 20 ug protein derived from infected samples. Similar results were observed when comparing BHV-1 and HSV-1 infection in the human osteosarcoma cell line (U2OS) that is permissive for both viruses (data not shown).

Additional studies were performed to examine E2F1 protein levels in primary human embryonic lung (HEL) fibroblasts after infection with HSV-1. The rationale for this study was to compare the results obtained in RS cells to a low passage human cell type. Confluent cultures of HEL fibroblasts were infected with HSV-1 at a moi of 1 and whole cell lysate collected at the designated times (hours) after infection. In HEL cells, HSV-1 infection increased the steady state E2F1 protein levels at 16 hours after infection (Figure 5A). CIAP treatment resulted in a decrease in the electrophoretic mobility of E2F1 in HEL cells (Figure 5B). In summary, both BHV-1 and HSV-1 increased E2F1 protein levels, and HSV-1 infection led to novel changes in the phosphorylation status of E2F1 in RS and HEL cells.

Analysis of E2F1 localization following infection

A previous study revealed that E2F1 remained localized to the nucleus following infection of bovine kidney cells with BHV-1 (Workman and Jones, 2010). Conversely, E2F1 was reported to localize to the cytoplasm following HSV-1 infection of HeLa cells (Advani *et al.*, 2000). Confocal microscopy suggested that a subset of E2F1 was localized to the cytoplasm after infection of RS cells with HSV-1 (data not shown). To confirm these findings and compare them to BHV-1 productive infection, biochemical fractionation studies were conducted in RS cells. Two additional moi, 0.1 or 0.5 pfu/cell, were used for the HSV-1 studies. A moi of 5 pfu/cell was used for BHV-1 because BHV-1 does not grow as quickly as HSV-1 in RS cells (data not shown). Consistent with previous studies (Workman and Jones, 2010), E2F1 was detected in the nuclear extract, but not the cytoplasm, of BHV-1 infected cells at 16 or 24 hours after infection (Figure 6A). At a lower moi, E2F1 was also detected in the nucleus of RS cells infected with BHV-1 (data not shown). Alternatively, E2F1 was detected in the nuclear and cytoplasmic extract following infection with HSV-1 for 16 or 24 hours regardless of whether the moi was 0.1 or 0.5 (Figure 6B). Eight hours after HSV-1 infection (moi of 0.1 and 0.5 pfu/ml) E2F1 was primarily detected in the nucleus of infected cells. As a control for nuclear proteins, we examined histone H3 and found, as expected, that histone H3 was detected only in the nucleus after infection of RS cells with HSV-1 or BHV-1 (Figure 6A and B).

Analysis of E2F-dependent transcription following infection

The studies in Figures 3–6 indicated that HSV-1 and BHV-1 increased E2F1 protein levels, but these studies did not reveal whether E2F dependent transcription correlated with increased E2F1 protein levels. Therefore, we examined the effect of BHV-1 or HSV-1 productive infection on E2F dependent transcription. RS cells were initially transfected with a luciferase construct containing a minimal promoter with three consensus E2F binding sites (3xE2F) or the Cyclin D promoter (CycD). Cells were then infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5 for 24 hours. BHV-1 infection increased 3xE2F promoter activity approximately 5 fold and CycD promoter activity approximately 4 fold in RS cells (Figure 7). In contrast, HSV-1 had little or no effect on the 3xE2F promoter construct, and CycD promoter activity was consistently reduced. These results indicated that E2F dependent transcription was stimulated following infection of RS cells with BHV-1, but not HSV-1.

DISCUSSION

In this study, we compared the effect that BHV-1 had on the cell cycle regulated transcription factor E2F1 versus HSV-1. For these studies, we used a spontaneously immortalized rabbit skin cell line that supports productive infection of both viruses. The studies clearly demonstrated that a siRNA directed against E2F1 reduced the plaque forming efficiency of HSV-1 (Figure 1) and BHV-1 (Workman and Jones, 2010). For HSV-1, this was somewhat surprising because E2F dependent transcription was not activated after infection. In this study and a previous study, we demonstrated that BHV-1 infection induced

E2F dependent transcription and binding to a probe containing E2F consensus binding sites (Workman and Jones, 2010). We suggest that silencing E2F1 prior to infection reduces the number of cells that are cycling, which consequently reduces the efficiency of productive infection. This conclusion is supported by other studies demonstrating that cyclin dependent kinase inhibitors, roscovitine and olomucine, reduce HSV-1 virus replication and transcription (Schang *et al.*, 1999; Schang *et al.*, 1998). Roscovitine also efficiently reduces BHV-1 productive infection (data not shown).

Following infection with HSV-1, E2F1 appeared to be differentially phosphorylated and a fraction of the total E2F1 protein was localized in the cytoplasmic. In contrast, E2F1 was localized to the nucleus after BHV-1 infection, as judged by biochemical fractionation (Figure 6) and confocal microscopy (Workman and Jones, 2010). Since HSV-1 inactivated E2F dependent transcription, we suggest that phosphorylation of E2F1 promotes localization of E2F1 to the cytoplasm, resulting in a loss of E2F dependent transcription. Inactivating E2F1 by HSV-1 may be important for efficient productive infection because E2F1 induces apoptosis following DNA damage (Nip *et al.*, 1997). HSV-1 encodes two protein kinases, US3 and UL13 that are packaged in the virion and tegument respectively (Chee *et al.*, 1989; Frame *et al.*, 1987; Purves *et al.*, 1987; Smith and Smith, 1989). Plasmids over-expressing the two viral kinases were cotransfected with E2F1 and there was no obvious changes in the mobility of E2F1 (data not shown) suggesting the viral encoded protein kinases do not directly phosphorylate E2F1. E2F1 is directly phosphorylated by several cellular protein kinases, including glycogen synthase kinase-3 β , ATM (ataxia telangiectasia mutated), and mitogen activated protein kinases (MAPK) (Garcia-Alvarez *et al.*, 2007; Real *et al.*, 2010; Weei-Chin *et al.*, 2011). HSV-1 activates MAPK and ATM during productive infection (Boutell and Everett, 2004; Corcoran *et al.*, 2006; Hargett *et al.*, 2005) suggesting E2F1 was phosphorylated by one of these cellular protein kinases. It is not known whether BHV-1 infection induces MAPK or ATM protein kinases during the course of productive infection.

Transcriptionally competent E2F family members are associated with a family of DP proteins (Helin *et al.*, 1993) suggesting BHV-1 stimulates E2F dependent transcriptional activity by promoting interactions between E2F1 and DP family member. Conversely, HSV-1 may uncouple E2F1 dependent transcription by inactivating DP family members. A recent study identified a novel DP family member (DP-4) in several human cell types that exhibits high levels of expression following DNA damage (Ingram *et al.*, 2011). Interestingly, DP-4 binds to E2F1 resulting in a complex that does not bind DNA. Consequently, E2F1 does not induce apoptosis when associated with DP-4. There is no commercially available DP-4 antibody, thus it is not possible to test whether DP-4 is induced by HSV-1 infection in RS cells, or whether BHV-1 induces DP-4. It will be of interest to examine the effect that HSV-1 has on DP-4 in human cells.

In summary, it was surprising to find that E2F dependent transcription was activated by BHV-1, but not HSV-1. Activation of E2F dependent transcription appears to be important for BHV-1 productive infection because E2F1 and E2F2 strongly stimulate the bICP0 E promoter and to a lesser extent the IE promoter that controls expression of bICP0 and bICP4 (Geiser and Jones, 2003; Workman and Jones, 2010). We suggest that BHV-1 encodes or induces a protein that maintains E2F1 transcriptional activity during productive infection. Conversely, HSV-1 may encode or induce a factor that prevents activation of E2F dependent transcription during productive infection.

METHODS

Cells and viruses

Murine neuroblastoma 2A (neuro-2A), human embryonic lung (HEL), and rabbit skin (RS) cells were grown in Earle's modified Eagle's medium (EMEM) supplemented with 5% fetal calf serum (FCS). Bovine kidney cells (CRIB) were grown in EMEM supplemented with 10% FCS. All media contained penicillin (10 U/ml) and streptomycin (100 µg/ml).

The Cooper strain of BHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. Stock cultures of BHV-1 were prepared in CRIB cells.

The HSV-1 McKrae strain was obtained from S. Wechsler (U of California-Irvine). Stock cultures of the McKrae strain were prepared in RS cells.

Plasmids and measurement of promoter activity in transfected cells

Plasmids expressing E2F1 or E2F2, pCMV-E2F1 and pCMV-E2F2 respectively, were obtained from J.R. Nevins (Duke University, Durham, USA). CAT constructs used in this study, HSV-1 ICP0-CAT (pAB5) and ICP4-CAT, were previously described (Devireddy and Jones, 2000). Luciferase promoter constructs used in this study, pGL3basic, VP16-luc, gC-Luc, UL42-Luc, and ICP6-Luc, were obtained from P. Schaffer and have been previously described (Kushnir, 2010). The empty vector pcDNA3.1 was purchased from Invitrogen.

Measurement of chloramphenicol acetyltransferase (CAT) activity in neuro-2A cells was performed as described previously (Workman *et al.*, 2011; 2009; Workman and Jones, 2010). Luciferase activity in transfected neuro-2A cells was performed using the Promega Luciferase Assay System as described by the manufacturer (E4030; Promega).

For productive infection assays, RS cells were transfected with an E2F-responsive promoter construct using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours later, cells were infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5 for 24 hours. Cells were then collected and processed as above for luciferase activity. Data for CAT and luciferase activity were averaged from the results of multiple transfections performed in at least three independent experiments.

SDS-polyacrylamide gels and Western Blotting of E2F Expression

RS cells or HEL cells were infected with wt BHV-1 or HSV-1 at the moi indicated in the respective figure legend and cell lysate collected at various times (hours) after infection. Whole cell lysate was prepared as described previously (Workman *et al.*, 2011; Workman and Jones, 2010), and protein concentrations were quantified by the Bradford assay. Standard 12% SDS-polyacrylamide gels were used to analyze E2F1 protein levels as described previously (Workman and Jones, 2010). The E2F1 antibody (sc-193X; Santa Cruz Biotechnology) was diluted 1:10,000 in blocking solution. An antibody directed against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control.

Nuclear and Cytoplasmic Fractionation

RS cells were infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5 for 8, 16 or 24 hours. Cells were harvested by centrifugation at 1,000 rpm for 5 min and washed twice in cold phosphate buffered saline (PBS). Cells were then suspended in buffer A (50 mM NaCl, 10 mM HEPES pH8, 500 mM sucrose, 1 mM EDTA, 0.5% NP40), vortexed briefly, and centrifuged at 5,000-x g for 2 minutes. The supernatant was collected and saved as the

cytoplasmic fraction. The nuclear pellet was washed twice with low salt buffer B (50 mM NaCl, 10 mM HEPES pH8, 25% glycerol, 0.1 mM EDTA). The pellet was then suspended in high salt lysis buffer C (350 mM NaCl, 10 mM HEPES pH8, 25% glycerol, 0.1 mM EDTA). Lysis was carried out at 4° C for 30 min. Supernatant was cleared by centrifuging at 13,000 rpm for 15 minutes at 4° C. Protein concentrations were quantified by the Bradford assay and standard 8% SDS-polyacrylamide gels were used as described above to analyze E2F1 protein levels. An antibody directed against histone H3 (Abcam; ab1191) was diluted 1:500 and used as a control for fractionation studies.

Analysis of HSV-1 productive infection

RS cells grown in 60 mM dishes were transfected with 1 µg of blank pcDNA3.1 vector, 100 nM E2F1 siRNA (sc-35247; Santa Cruz Biotechnology) or 100 nM control siRNA (44-2926; Invitrogen) using Lipofectamine 2000 according to the manufacture's specifications. At 24 hours after transfection, cells were transfected with 2 µg of the HSV-1 viral genome. Forty-eight hours after transfection cells were collected and subjected to three rounds of freeze/thaw cycles. Cell debris was pelleted by centrifugation, and the supernatant was analyzed by plaque assay on RS cells using 10 fold dilutions.

Cell Cycle Analysis by Flow Cytometry using the Telford Method

RS cells grown in 60 mM dishes were transfected with 100 nM E2F1 siRNA or a control siRNA using Lipofectamine-2000 according to the manufacture's specifications. At 24 hours or 48 hours after transfection, an aliquot of 1×10^6 RS cells were pelleted, then suspended in 1 mL of ice-cold 70% ethanol, and rotated at 4 degrees for 30 minutes. Cells were then pelleted and suspended in Telford reagent (100 mM EDTA, 0.1% TritonX-100, 75 mM Propidium Iodide, and 2.5 U/mL RNase A in PBS pH 7.4) and rotated at 4 degrees for 2 hours. Cells were then analyzed using a Becton Dickson FACS caliber flow cytometer. The data was analyzed using Cell Quest.

Alkaline Phosphatase (CIAP) treatment

Whole cell lysate was collected as described above. Twenty µg of infected cell lysate was treated with 10 U of alkaline phosphatase (04 898 133 001; Roche) and incubated at 37 degrees for 30 minutes. Reactions were stopped by the addition of SDS-polyacrylamide gel loading buffer. Samples were boiled for 5 minutes and assayed by immunoblotting.

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Highlights

This study provides evidence that replication of herpes simplex virus type 1 (HSV-1) and bovine herpesvirus (BHV-1) was reduced when E2F1 was silenced. Furthermore, both viruses increased the protein levels of E2F1. BHV-1 productive infection, but not HSV-1 productive infection, induced E2F1 dependent transcription. In conclusion, these studies demonstrated that HSV-1 and BHV-1 have different effects on E2F1 during productive infection.

Figure 1. Suppression of E2F1 reduced the levels of productive infection

Panel A: To test whether E2F-specific siRNA affected HSV-1 productive infection, RS cells were transfected with 100 nM E2F1 siRNA or 100 nM control siRNA for 24 hours, followed by transfection of HSV-1 genomic DNA as described in the methods. Forty-eight hours after transfection of genomic DNA cells were collected, subjected to three rounds of freeze/thaw cycles and plaque assays performed on RS cells. The data is an average of three independent studies and the error bars are standard deviations.

Panel B: To examine the effect of the siRNAs on cell cycle, RS cells were transfected with 100 nM E2F1 siRNA or 100 nM control siRNA. At 24 or 48 hours after transfection, cells were collected, fixed, stained and analyzed by flow cytometry as described in the materials and methods section. These studies are representative of at least three independent studies.

Figure 2. Analysis of E2F1 transactivation of HSV-1 promoter constructs

Panel A: To examine the effect of E2F1 on HSV-1 promoters, neuro-2A cells were cotransfected with 1 μ g of the designated promoter-CAT construct and 0.1 μ g of the E2F1 expression plasmid. At 48 hours post-transfection, cells were collected and CAT activity measured. The data is an average of three independent studies and the error bars are standard deviations.

Panel B: To test whether silencing E2F1 protein levels reduced basal activity of HSV-1 promoters, neuro-2A cells were cotransfected with 1 μ g of the designated promoter-CAT construct and 100 nM E2F1 siRNA or a control siRNA (contsi). At 48 hours post-transfection, cells were collected and processed for CAT activity.

Panel C: Neuro-2A cells were cotransfected with 1 μ g of the designated promoter-luciferase construct and 0.1 μ g of the E2F1 expression plasmid. At 48 hours post-transfection, cells were collected and processed for luciferase expression. The data is an average of three independent studies and the error bars are standard deviations.

Figure 3. Analysis of E2F1 protein levels during productive infection

The effect of BHV-1 or HSV-1 infection on E2F1 protein levels was measured by western blot analysis. RS cells were infected with BHV-1 (moi =5; **Panel A**), HSV-1 at an (moi = 5; **Panel B**), or HSV-1 at a moi of 0.5 (**Panel C**) for the designated times after infection (hours). Cells were collected, lysed, and 100 μ g of protein was analyzed by western blot using an E2F1 polyclonal antibody diluted 1:10,000. As a control, the blot was also probed with antiserum against β -actin. Lanes M4 or M24 are 4 or 24 hours respectively after mock infection.

Figure 4. Analysis of E2F1 protein levels in infected RS cells

Panel A: Cell lysate prepared from RS cells infected with BHV-1 (moi = 5) or HSV-1 (moi = 5) for 24 hours were separated on the same SDS-polyacrylamide gel to show mobility differences of E2F1.

Panel B: To determine whether phosphorylation was responsible for the altered mobility of E2F1, 20 µg of protein derived from cell lysate prepared from RS cells infected with HSV-1 for 24 hours was treated (or not) with calf intestinal alkaline phosphatase (CIAP). As a control, 200 µg of mock-infected cells was analyzed. More protein was loaded in the mock lane to visualize the E2F1-specific bands.

Figure 5. Analysis of E2F1 protein levels in normal low passage human lung fibroblasts

Panel A: Low passage human embryonic lung fibroblasts (HEL) were infected with HSV-1 at a moi of 1 for the designated times after infection (hours). Cells were collected, lysed, and 100 µg of protein analyzed by western blot using an E2F1 polyclonal antibody diluted 1:10,000. As a control, the blot was also probed with antiserum directed against β-actin.

Panel B: Twenty µg of protein from the sample at 24 hours after infection of HEL cells were treated (or not) with calf intestinal alkaline phosphatase (CIAP) and ran next to 200 µg of cell lysate from mock-infected cells. More protein was loaded in the mock-infected lane (lane M) to visualize E2F1-specific bands.

Figure 6. Localization of E2F1 after infection with BHV-1 or HSV-1

Panel A: RS cells were infected with BHV-1 at a moi of 5 for 8, 16, or 24 hours. Cells were then harvested and cell fractionation conducted as described in the methods section.

Cytoplasmic and nuclear fractions (100 μ g) were analyzed by western blot using an E2F1 polyclonal antibody diluted 1:10,000. As a control for proteins that are in the nucleus, the respective fractions were probed with an antibody directed against Histone H3 that was diluted 1:500.

B. RS cells were infected with HSV-1 at a moi of 0.1 or 0.5 for 8 or 16 hours. Cellular fractions were analyzed as described in panel A. Lane M was cell lysate derived from mock-infected cells.

Figure 7. Activity of E2F-responsive promoters following BHV-1 or HSV-1 infection

RS cells were transfected with 1 μ g of the designated promoter-luciferase construct (a promoter containing 3 consensus E2F1 binding sites: 3xE2F or the cyclin D promoter: CycD). Twenty-four hours after transfection, cells were infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5. At 24 hours after infection, cell lysate was collected and luciferase activity measured. The results are the average of 3 independent experiments. The data is an average of three independent studies and the error bars are standard deviations.