Stimulation of bovine herpesvirus-1 productive infection by the adenovirus E1A gene and a cell cycle regulatory gene, E2F-4

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Identifying cellular genes that promote bovine herpesvirus-1 (BHV-1) productive infection is important, as BHV-1 is a significant bovine pathogen. Previous studies demonstrated that BHV-1 DNA is not very infectious unless cotransfected with a plasmid expressing bICP0, a viral protein that stimulates expression of all classes of viral promoters. Based on these and other studies, we hypothesize that the ability of bICP0 to interact with and modify the function of cellular proteins stimulates virus transcription. If this prediction is correct, cellular proteins that activate virus transcription could, in part, substitute for bICP0 functions. The adenovirus E1A gene and bICP0 encode proteins that are potent activators of viral gene expression, they do not specifically bind DNA and both proteins interact with chromatin-remodelling enzymes. Because of these functional similarities, E1A was tested initially to see if it could stimulate BHV-1 productive infection. E1A consistently stimulates BHV-1 productive infection, but not as efficiently as bICP0. The ability of E1A to bind Rb family members plays a role in stimulating productive infection, suggesting that E2F family members activate productive infection. E2F-4, but not E2F-1, E2F-2 or E2F-5, activates productive infection with similar efficiency as E1A. Next, E2F family members were examined for their ability to activate the BHV-1 immediate-early (IE) transcription unit 1 (IEtu1) promoter, as it regulates IE expression of bICP0 and bICP4. E2F-1 and E2F-2 strongly activate the IEtu1 promoter, but not a BHV-1 IEtu2 promoter or a herpes simplex virus type 1 IC0 promoter construct. These studies suggest that E2F family members can stimulate BHV-1 productive infection.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is an important virus pathogen of cattle that can cause severe respiratory infection, conjunctivitis, abortions, vulvovaginitis, balanopostitis and systemic infection in neonate calves (Jones, 1998). BHV-1 infection also initiates an upper respiratory tract infection, referred to as ‘shipping fever’, by immunosuppressing infected cattle, which results in secondary bacterial infections and pneumonia. (Tikoo et al., 1995). Increased susceptibility to secondary infection correlates with depressed cell-mediated immunity after BHV-1 infection (Carter et al., 1989; Griebel et al., 1987a, b, 1990). CD8+ T cell recognition of infected cells is impaired by repressing expression of major histocompatibility complex class I and the transporter associated with antigen presentation (Hariharan et al., 1993; Hinkley et al., 1998; Nataraj et al., 1997). CD4+ T cell function is impaired during acute infection of calves, in part, because BHV-1 infects CD4+ T cells and induces apoptosis (Winkler et al., 1999). BHV-1 infection costs the cattle industry millions of dollars per year in the United States (Bowland & Shewen, 2000). Although modiﬁed live vaccines are available, they can cause disease in young calves or abortions in cows and they have the potential to establish latency and reactivate from latency (Jones et al., 2000).

BHV-1 is a member of the subfamily Alphaherpesvirinae and shares certain biological properties with herpes simplex virus types 1 and 2 (HSV-1 and -2) (Jones, 1998). Viral gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E) or late (L). Two IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2. IEtu1 encodes functional homologues of two HSV-1 proteins, ICP0 and ICP4. bICP0 is very important for productive infection because it activates all classes of viral promoters and is expressed at high levels throughout infection (Fraefel et al., 1994; Wirth et al., 1991, 1992).

Most alphaherpesviruses encode an ICP0-like transcription activator that contains a well-conserved C3HC4 zinc ring finger located near the N terminus of these proteins (Everett, 1988, 2000; Everett et al., 1993, 1995; Lium & Silverstein, 1997). These ICP0 homologues transactivate all classes of viral genes (Bowles et al., 1997; Fraefel et al., 1994; Koppel et al., 1996, 1997; Lium et al., 1998) demonstrating they are promiscuous transactivators. Mutational analysis has demonstrated the importance of the zinc ring finger
domain in HSV-1 ICP0 (Everett et al., 1993, 1995; Everett, 1988, 2000; Lium & Silverstein, 1997), equine herpesvirus-1 ICP0-like protein (Bowles et al., 1997, 2000) and BHV-1 bICP0 (Inman et al., 2001b). ICP0 (Everett et al., 1997, 1999a, b; Maul & Everett, 1994; Maul et al., 1993) and bICP0 (Inman et al., 2001b; Parkinson & Everett, 2000) colocalize with and disrupt the proto-oncogene promyelocytic leukemia protein-containing nuclear domains. ICP0 can regulate the stability of cellular and viral proteins by interacting with protein degradation machinery (Everett et al., 1997, 1999a). For example, the stability of the catalytic subunit of DNA-dependent protein kinase is regulated by ICP0 (Lees-Miller et al., 1996; Parkinson et al., 1999). ICP0 also binds cyclin D3 (Kawaguchi et al., 1997b) and elongation factor δ (Kawaguchi et al., 1997a). The results of these interactions are perturbation of the cell cycle and altered cellular gene expression (p21, gadd45 and mdm-2, for example) (Hobbs & DeLuca, 1999). Interestingly, a histone deacetylase inhibitor trichostatin A and ICP0 have similar effects on cellular and viral gene expression (Hobbs & DeLuca, 1999). bICP0 interacts with histone deacetylase 1, which correlates with activation of gene expression (Zhang & Jones, 2001). Consequently, regulating chromatin-remodelling enzymes may be an important mechanism by which bICP0 and the other ICP0 homologues regulate virus transcription. Unlike many transactivators of viral gene expression, the ICP0 homologues do not appear to specifically bind to DNA.

In transient transfection assays, BHV-1 DNA yield low numbers of plaques per μg DNA and there is a long latency period prior to plaque formation. Cotransfection of BHV-1 DNA with the gene encoding bICP0 or HSV-1 ICP0 increases the number of plaques and reduces the latency period prior to the appearance of plaques (Geiser et al., 2002; Inman et al., 2001a, b). Since bICP0 is a potent transactivator, we assumed that activation of virus transcription was the mechanism for stimulating plaque formation following transfection of cells with viral DNA. These observations also suggested that cellular genes that stimulate virus transcription can substitute for bICP0 or HSV-1 ICP0.

The adenovirus-encoded E1A protein has certain functions that appear to be similar to bICP0. For example, E1A creates a permissive environment for adenovirus productive infection, strongly activates virus transcription, interacts with chromatin-remodelling enzymes but does not directly bind DNA (White, 1998). In addition, E1A (White, 1998), like bICP0 (Inman et al., 2001b), is cytotoxic in transiently transfected cells. Although E1A clearly induces apoptosis, it is not clear how bICP0 kills cells. E1A cDNAs encode either a 289 or 243 aa protein that can cooperate with other oncogenes to transform primary baby rat kidney cells (Zerler et al., 1986). The E1A protein contains several domains that regulate different aspects of the cell cycle, transcription activation and apoptosis. Through mutational analysis, the specific functions of these domains have been identified. Two conserved regions (CR1 and CR2) are required for interaction with p300 and the Rb family of pocket proteins (Rb, p107 and p130) (Stein et al., 1990; Wang et al., 1993). Immortalization of mammalian cells by E1A is mediated independently by two regions of the protein that bind p300 and Rb pocket proteins.

The interaction of E1A with a Rb family member leads to the release of bound E2F transcription factors (Harbour & Dean, 2000). In general, E2F proteins that are bound to Rb pocket proteins repress transcription, whereas unbound E2F activates transcription. The association of Rb with histone deacetylases plays an important role in repressing E2F transcription activity. The family of E2F transcription factors interacts with DP (differentiation-regulated transcription factor 1 protein) family members, which are necessary for E2F transcription activation. E2F proteins contain a conserved DNA-binding domain, an acidic transcription activation domain and a Rb-binding site (Harbour & Dean, 2000). Functional E2F-binding sites are present in the promoters of genes controlling cell cycle progression (DeGregori et al., 1995; Nevins et al., 1997; Ohtani et al., 1995; Schulze et al., 1995; Wells et al., 1997). There are several E2F family members and it is clear that these different family members have novel biological properties, in addition to binding consensus E2F-binding sites (Harbour & Dean, 2000).

In this study, we tested whether non-BHV-1 genes could replace bICP0 and activate productive infection following transfection of bovine cells. The adenovirus E1A gene and E2F-4 gene stimulated productive BHV-1 infection. In contrast, E2F-1, E2F-2 and E2F-5 had little or no effect on productive infection. Since the BHV-1 IEtu1 promoter regulates bICP0 expression, we tested whether the E2F genes could activate IEtu1 promoter activity. E2F-1 and E2F-2 activated IEtu1 promoter activity more than 10-fold in transient transfection assays. In contrast to the IEtu1 promoter, E2F family members did not transactivate the BHV-1 IEtu2 promoter nor an HSV-1 ICP0 promoter construct. In summary, these results implied that distinct E2F family members have the potential to regulate productive infection.

**METHODS**

**Plasmid constructs used in this study.** The construction and characteristics of pcMV-bICP0 (bICP0) has been described previously (Inman et al., 2001b). The adenovirus E1A constructs (12S.WT, 12S.RG2, 12S.YH47, 12S.928, 12S.RG2.928 and 12S.YH47.928) were obtained from E. Moran (Cold Spring Harbor, NY, USA) and have been described previously (Dallas et al., 2000; Wang et al., 1991, 1993) (for a schematic of these constructs, see Fig. 1B). Plasmids expressing E2F family members, pCMV-E2F1 (E2F1), pCMV-E2F-2 (E2F-2), pC4DNA-E2F-4 (E2F-4) and pC4DNA-E2F5 (E2F5) were obtained from J. R. Nevins (Durham, NC, USA). Plasmids encoding VP16 and CAT used in this study, pAB5 (HSV-1 ICP0cat) (Devireddy & Jones, 2000), p17380CAT (BHV-1 IEtu2cat) (Schwyzer et al., 1994), pE1 (IEtucat), pE1A Δ31 (IEtucatΔ31), pE1ΔΔ1018 (IEtucatΔ1018) and pE1ΔΔ1766
Fig. 1. Regulation of productive infection by wt and mutant E1A 12S-encoding plasmids. (A) Regulation of productive infection by wt adenovirus E1A 12S and various mutants. BFL cells were cotransfected using Superfect with 1 µg gC blue virus genomic DNA and 4 µg of the respective adenovirus E1A 12S plasmid. bICP0 was used as a positive control for transactivation levels. At 36 h after transfection, cells were fixed, stained and the number of blue cells counted. The number of β-Gal+ cells in the vector control (pUC19) was set at 1-fold and the number of β-Gal+ cells in each well was calculated as the fold of the vector control. The results are the average of three independent experiments. (B) Schematic of the adenovirus E1A protein and the mutants used for this study. A mutation of arginine to glycine at aa 2 (12S.RG2) yields an E1A protein that does not bind p300 and induce p53 levels (Wang et al., 1991, 1993). The 12S.YH47 mutation encodes a protein that does not bind Rb and p130. A mutation containing a deletion of nt 928–961 (12S.928) encodes a protein that does not bind p130 and exhibits reduced binding to p107. Mutations at position 928 and 2 (12S.RG2.928) encode a protein that does not bind Rb, p130, and p107. Mutations at positions 928 and 47 (12S.YH47.928) encode a protein that does not bind Rb and p130.

**Cells and transfections.** Bovine kidney (MDBK) cells were plated at a density of 5 x 10^5 cells per 100 mm^2 plastic dish in Earle's modified Eagle's medium supplemented with 5% FBS, penicillin (10 U ml^-1) and streptomycin (100 µg ml^-1). MDBK cells were split at a 1:8 ratio every 3 days. Low passage bovine foetal lung (BFL) cells were obtained from C. Wood (Lincoln, NE, USA). BFL cells were split at a ratio of 1:4 every 3 days and plated as described for MDBK cells, except that BFL cells were supplemented with 10% FBS.

BFL cells were transfected with the designated plasmids, as described by the manufacturer, using Superfect (Qiagen). At 16 h prior to transfection, 1 x 10^6 cells were plated into each well of a 6-well plate or 4 x 10^5 cells per 60 mm dish. The respective plasmids and viral genomic DNA in the indicated amounts were added together. The total quantity of DNA was adjusted using pcDNA3.1- or pUC19 vector control to 3 µg per well (5 µg per 60 mm dish). The total volume was adjusted to 100 µl (300 µl per 60 mm dish) by adding media without serum or antibiotics followed by 12 µl (20 µl per 60 mm dish) of Superfect. The solution was incubated at room temperature for 10 min and then the cells were rinsed with calcium- and magnesium-free PBS (CMF-PBS); 900 µl (1-4 ml per 60 mm dish) of media with serum and antibiotics was then added to the DNA/Superfect solution and this added to the cells. After 3 h at 37°C, cultures were rinsed three times with CMF-PBS and fresh media added.

**Virus.** A BHV-1 mutant containing the β-galactosidase (β-Gal) gene in place of the viral gC gene was obtained from S. Chowdury (Manhattan, KS, USA) (gC blue virus). The virus grows to similar titres as the wild-type parent virus and expresses the β-Gal gene.

**Extraction of viral genomic DNA.** The procedures for preparing BHV-1 genomic DNA have been described previously (Geiser et al., 2002; Inman et al., 2001a, b, 2002).

**β-Gal assay.** β-Gal activity was measured at 24 or 36 h after transfection, as described previously (Geiser et al., 2002). The number of β-Gal+ cells in cultures cotransfected with the optimal concentration of bICP0 and viral genomic DNA was set at 100% infectivity for each experiment. This representation of data minimized the differences in cell density, Superfect lot variation and transfection efficiency.

**CAT assays.** Cells were cotransfected with the designated plasmids as indicated. Cells were harvested 48 h after transfection and the levels of CAT were measured as described previously (Delhon & Jones, 1997). After thin layer chromatography, a Phosphorimager (Molecular Dynamics) was used to determine the amount of acetylated or unacetylated [3H]Chloramphenicol (CM). The per cent acetylation of cells in the vector control (pcDNA3.1–) was set at 1-fold and the per cent acetylation of each sample was calculated relative to the vector control. This representation of data minimized the differences in cell density, Superfect lot variation and transfection efficiency.

**RESULTS**

**Adenovirus E1A gene activates productive infection**

Transfection of bovine cells with BHV-1 genomic DNA yields low levels of infectious virus. Cotransfection of BHV-1 DNA with a plasmid expressing bICP0 enhances productive infection and virus yield (Inman et al., 2001b). As discussed above, E1A has certain functional features that resemble bICP0. To test whether E1A could stimulate productive BHV-1 infection, BHV-1 genomic DNA and a
plasmid expressing the wild-type (wt) E1A 12S protein (12S.WT) were cotransfected into low passage BFL cells and productive infection was measured. A BHV-1 mutant that contains the β-Gal gene inserted downstream of the gC gene (gC blue virus) was used for this study. At 36 h after transfection, cells were fixed and assayed for β-Gal activity. This time-point was used as the time to count β-Gal+ cells to minimize the number of virus-positive cells that result from virus spread. At later times, many of the β-Gal+ cells lifted off the dish, making it difficult to accurately count virus-positive cells (Geiser et al., 2002; Inman et al., 2001b). The number of β-Gal+ cells correlates directly with the number of plaques produced following transfection with the gC blue virus (Inman et al., 2001b; data not shown).

When a bICP0-expressing plasmid was cotransfected with BHV-1 DNA, the number of β-Gal+ cells increased approximately 5-fold (Fig. 1A), which was consistent with our previous studies (Inman et al., 2001b). When the E1A 12S.WT expression plasmid was cotransfected with BHV-1 DNA, the number of β-Gal+ cells increased more than 3-fold (Fig. 1A), indicating that the E1A gene activated BHV-1 productive infection.

Several E1A mutants have been constructed to identify domains that regulate cell cycle and gene expression. The 12S.WT plasmid is the wt adenovirus E1A construct that all mutants were derived from. The mutants used in this study are described in Methods and are summarized schematically in Fig. 1(B). To determine what effects these mutations had on stimulating productive infection, the respective E1A mutants were cotransfected with BHV-1 DNA into BFL cells and the number of β-Gal+ cells counted at 36 h after transient transfection. The 12S.RG2 mutant encodes a protein that does not bind p300 or induce p53 expression and which stimulated BHV-1 productive infection with similar efficiency as wt E1A (Fig. 1A). A mutation at position 47 (12S.YH47) encodes a protein that does not bind Rb and p130 and this mutant stimulated productive infection less efficiently than wt E1A. A mutation at position 928 (12S.928), or in combination with aa 2 (12S.RG2.928) or 47 (12S.YH47.928), also reduced the number of β-Gal+ cells. Although it was clear that none of the mutants completely prevented stimulation of productive infection, these studies suggested that the ability of E1A to bind Rb family members played a role in stimulating productive infection.

**E2F-4 stimulates productive infection**

The underphosphorylated forms of the Rb family associate with E2F family members to repress proliferation, differentiation and apoptosis in a variety of tissues (Harbour & Dean, 2000). The interaction of E1A with the underphosphorylated forms of the Rb family leads to release of E2F family members. Consequently, we hypothesized that E2F family members may play a role in stimulating BHV-1 productive infection. To measure the effect of E2F on productive infection, increasing amounts of plasmids expressing E2F-1, E2F-2, E2F-4 or E2F-5 were cotransfected with BHV-1 genomic DNA and the number of β-Gal+ cells counted at 36 h after transfection. Cultures transfected with E2F-4 and BHV-1 DNA consistently increased the number of β-Gal+ cells more than 3-fold (Fig. 2). In contrast, cotransfection of BHV-1 DNA with E2F-1 or E2F-2 yielded similar numbers of β-Gal+ cells as those transfected with just the blank expression vector. E2F-5 increased the number of β-Gal+ cells approximately 2-fold. In summary, this study indicated that E2F-4 consistently stimulated BHV-1 productive infection by 3-fold, which was similar to wt E1A.

**E2F family members stimulate IEtu1 promoter activity**

Since E2F family members are transcriptional regulators, we tested whether any of the E2F constructs could activate the BHV-1 IEtu1 promoter, as this promoter regulates IE expression of bICP0 (Wirth et al., 1989, 1991, 1992). BFL cells were cotransfected with increasing amounts of a plasmid expressing one of the E2F family members and a CAT plasmid containing the IEtu1 promoter (IEtu1cat).

![Fig. 2. Stimulation of productive BHV-1 infection by plasmid constructs expressing E2F family members. BFL cells were cotransfected using Superfect with increasing amounts of plasmids expressing E2F family members (3-9, 15-6, 62-5 or 250 ng or 1 μg) and the gC blue virus genome (1 μg DNA). Because of the size differences in the E2F plasmids used and BHV-1 DNA, the ratios of plasmid DNA molecules to viral genomic DNA molecules are (1:4, 1:1, 4:1, 16:1 or 64:1), if one compares the number of molecules used for the transfection. Increasing concentrations of bICP0 plasmid (3-9, 15-6 and 62-5 ng) and 1 μg of the gC blue virus genome was used as a positive control for activation of productive infection. A blank expression vector (pcDNA3.1−) was used to maintain equivalent amounts of DNA. At 24 h after transfection, cells were fixed, stained and the number of blue cells counted. The number of β-Gal+ cells in the vector control (pcDNA3.1−) was set at 1-fold and the number of β-Gal+ in each well was calculated as the fold of the vector control. The results are the average of three independent experiments.](image-url)
Cultures cotransfected with a blank expression vector (pcDNA3.1−) and IEtu1cat were used to measure background levels of CAT activity. E2F-1 and E2F-2 consistently transactivated the IEtu1 promoter more than 10-fold, whereas E2F-4 and E2F-5 had no effect on IEtu1 promoter activity (Fig. 3). As demonstrated previously, VP16 transactivated the IEtu1 promoter more than 50-fold (Misra et al., 1994, 1995) but bICP0 had little effect on IEtu1 promoter activity (Wirth et al., 1992).

To localize DNA sequences within the IEtu1 promoter that were necessary for E2F1 transactivation, transient transfection assays were conducted using BHV-1 IEtu1 promoter deletion mutants. The IEtu1 promoter contains several cis-acting sequences that play a role in regulating promoter activity (Misra et al., 1994) (Fig. 4A). Increasing amounts of E2F-1 were cotransfected with one of the deletion promoter constructs. Although E2F-1 transactivated IEtu1catΔ831 and IEtu1catΔ1018 more than 5-fold, the levels of promoter activation was less than IEtu1cat (Fig. 4B). In contrast, transactivation of IEtu1catΔ1766 was only detected at the highest level of E2F-1 and transactivation was less than 5-fold. In summary, this study demonstrated that DNA sequences deleted from IEtu1catΔ1766 were important for E2F-1-mediated transactivation and suggested further that multiple elements within the IEtu1 promoter were responsive to E2F-1.

E2F-4 stimulates BHV-1 IEtu1 promoter

BHV-1 contains two IE transcription units, IEtu1 and IEtu2 (Wirth et al., 1991). Since E2F-1 and E2F-2 transactivated the IEtu1 promoter, we felt that it was important to test whether the other BHV-1 IE promoter (IEtu2) could be transactivated by E2F family members. To determine if IEtu2 was transactivated by E2F family members, increasing amounts of E2F family members, or bICP0, were cotransfected with a plasmid that contains the IEtu2 promoter region linked to the CAT reporter (IEtu2cat).
bICP0, which transactivated the BHV-1 IEtu2 promoter slightly (Fig. 5A), E2F-1 was not able to stimulate IEtu2 promoter activity. In addition, E2F-2, E2F-4 and E2F-5 did not activate IEtu2 promoter activity (data not shown).

To examine further the ability of E2F to activate herpesvirus promoters, E2F-1 and E2F-4 were cotransfected with an HSV-1 ICP0 promoter construct (pAB5). Using the same concentrations of E2F-1 that activated the IEtu1 promoter, we were unable to stimulate the ICP0 promoter (Fig. 5B). As expected, bICP0 and VP16 activated ICP0 promoter activity. In summary, E2F-1 did not stimulate the IEtu2 promoter nor an HSV-1 ICP0 promoter construct.

**DISCUSSION**

In this study, we tested whether non-BHV-1 genes could stimulate productive infection following transfection of BHV-1 DNA into bovine cells. BHV-1 has low plaquing efficiency following transfection of DNA, unless transfected with bICP0. The results presented in this study indicated that E1A and E2F-4, but not E2F-1, E2F-2 or E2F-5, stimulated productive BHV-1 infection. Stimulation of productive infection by E1A and E2F-4 was not as efficient as bICP0. We suggest that this approach can be used to identify other cellular genes that stimulate BHV-1 productive infection.

Since E2F-4 was the only E2F family member that was capable of consistently activating productive infection, it is reasonable to ask whether E2F-4 has novel properties or
functions. E2F-4 lacks a nuclear localization signal (Helin et al., 1992; Kaelin et al., 1997) and the cyclin A-binding site that is present at the N terminus of other E2F proteins (Harbour & Dean, 2000). E2F-4 transcription activity is regulated by phosphorylation (Helin et al., 1993; Tao et al., 1997), association with other cellular proteins (Weintraub et al., 1992) and subcellular localization (Allen et al., 1997; Verona et al., 1997). E2F-4 can bind to all of the Rb family members but there is a preference for p107 and p130 (Harbour & Dean, 2000). Ectopic expression of E2F-4 in Chinese hamster cells does not activate E2F-dependent transcription, demonstrating that E2F-4 has novel functions (Chang et al., 2000). Interestingly, human immunodeficiency virus-encoded Tat specifically interacts with E2F-4 and Tat can stimulate an E2F responsive promoter (Ambrosino et al., 2002). Since E2F-4 does not directly stimulate IEtu1 promoter activity, we suggest that a virus-specific modification of E2F-4 may occur during productive infection that is necessary for IEtu1 promoter activation or E2F-4 activates productive infection by an independent mechanism.

Our studies have suggested that E2F family members, and perhaps other cell cycle regulators, stimulate BHV-1 productive infection. There are a number of studies that have concluded that cell cycle regulatory proteins play an important role in the early stages of infection. For example, it is clear that cell factors in G1 or S enhance the growth of ICP0− mutants (Cai & Schaffer, 1991) and the ability of VP16 to activate transcription (Daksis & Preston, 1992). Further support that cell cycle regulatory proteins play a role in productive infection comes from studies demonstrating that a Cdk2 and Cdc2 inhibitor (roscovitine) inhibits HSV-1 infection, in part because IE and E transcription is blocked (Schang et al., 1998, 1999). Although it is clear that infection with HSV-1 and presumably BHV-1 leads to cell cycle arrest (reviewed by Flemington, 2001), specific cell cycle regulatory proteins may stimulate productive infection in certain cell types.

Several studies have demonstrated that E2F function is modified following HSV-1 infection. For example, E2F not bound to Rb family members is increased following infection of human cells (C33-A) (Hilton et al., 1995). Relocalization of E2F-4 to the nucleus occurs in C33-A and U2-OS human cells following infection of HSV-1 (Olgiate et al., 1999). Further support for E2F-4 playing a role in HSV-1 replication comes from the findings that infection of p107−/−/p130−/− mouse cells leads to reduced titres of infectious virus (Ehmann et al., 2001). Since E2F-4 is bound to p107 and p130, this implies that E2F-4 plays a role in HSV-1 infection. Another study concluded that HSV-1 infection leads to inactivation of E2F family members. For example, in primary human fibroblasts or HeLa cells, the intracellular levels of E2F-4 are reorganized following HSV-1 infection, but this is assumed to inactivate E2F-4 activity (Advani et al., 2000). This same study also concluded that HSV-1 infections lead to post-translational modification of E2F-1 and E2F-5, translocation of E2F family members from the nucleus to the cytoplasm and reduced E2F binding to consensus E2F-binding sites. Since many DNA synthetic genes are activated by E2F family members (Harbour & Dean, 2000), we suggest that transient induction of one or another E2F family member could promote BHV-1 DNA synthesis in highly differentiated cells.

In spite of the fact that the IEtu1 promoter does not contain a consensus E2F-binding site, we believe that specific sequences in the IEtu1 promoter are responsive to E2F-1 and E2F-2. E2F-1 can activate the HSV-1 thymidine kinase promoter (Shin et al., 1996) and the ASK gene that encodes the regulatory subunit for human Cdc7-related kinase (Yamada et al., 2002), independently of a consensus E2F-binding site. The E2F responsive region of each promoter contains a GC-rich motif that resembles a Sp1-binding site. IEtu1 and IEtu2 promoters each contain numerous GC-rich sequences that resemble Sp1-binding sites, confirming further that activation of the IEtu1 promoter by E2F-1 and E2F-2 requires a novel motif that is only present in the IEtu1 promoter. It will be of interest to identify the specific cis-acting sequences in the IEtu1 promoter that are responsive to E2F-1 and determine what the effects of mutating these sequences have on productive infection and growth in cattle.

The following model is put forth to explain our findings with respect to BHV-1 biology and the potential role that E2F family members play in stimulating productive infection. The ability of E2F-4 to stimulate productive infection is proposed to be independent of activating the IEtu1 promoter. Additional studies are required to identify the step in productive infection that E2F-4 stimulates. Although E2F-1 did not dramatically stimulate BHV-1 productive infection, the ability of E2F-1 to activate the IEtu1 promoter has biological relevance during productive infection and perhaps reactivation from latency because G1 and S phase cyclins are activated in infected neurons during acute infection and reactivation from latency (Winkler et al., 1999). Since cyclin expression correlates with E2F transcription activation (reviewed by Harbour & Dean, 2000), the ability of BHV-1 to stimulate cyclin expression would lead to free E2F and activation of the IEtu1 promoter. Virion components of alpha/ herpesviruses can inhibit apoptosis (reviewed by Blaho & Aubert, 2001), suggesting that, in the context of virus infection, E2F-1 or E2F-2 could activate IEtu1 promoter activity without inducing apoptosis. Finally, this model predicts that BHV-1 productive infection leads to activation of E2F family members.

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