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Bovine herpesvirus 1 immediate-early protein (bICP0) interacts with the histone acetyltransferase p300, which stimulates productive infection and gC promoter activity

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Abstract: The immediate-early protein, bICP0, of *Bovine herpesvirus 1* (BHV-1) transactivates viral promoters and stimulates productive infection. bICP0 is expressed constitutively during productive infection, as its gene contains an immediate-early and an early promoter. Like other ICP0 homologues encoded by members of the subfamily *Alphaherpesvirinae*, bICP0 contains a zinc RING finger located near its N terminus. Mutations that disrupt the bICP0 zinc RING finger impair its ability to activate transcription, stimulate productive infection, inhibit interferon-dependent transcription in certain cell types and regulate subnuclear localization. bICP0 also interacts with a cellular chromatin-remodelling enzyme, histone deacetylase 1 (HDAC1), and can relieve HDAC1-mediated transcriptional repression, suggesting that bICP0 inhibits silencing of the viral genome. In this study, it was shown that bICP0 interacted with the histone acetyltransferase p300 during productive infection and in transiently transfected cells. In addition, p300 enhanced BHV-1 productive infection and transactivated a late viral promoter (gC). In contrast, a CH3-domain deletion mutant of p300, which is a dominant-negative mutant, did not activate the gC promoter. bICP0 and p300 cooperated to activate the gC promoter, suggesting that there is a synergistic effect on promoter activation. As p300 can activate certain antiviral signalling pathways (for example, interferon), it was hypothesized that interactions between p300 and bICP0 may dampen the antiviral response following infection.

INTRODUCTION

Infection of cattle with *Bovine herpesvirus 1* (BHV-1) can result in conjunctivitis, pneumonia, genital disorders, abortions and "shipping fever," an upper respiratory tract infection (Tikoo *et al.*, 1995). BHV-1 infection of bovine cells leads to rapid cell death and an increase in apoptosis (Devireddy & Jones, 1999). Viral gene expression is regulated in three distinct temporal phases: immediate-early (IE), early (E) and late (L) (Jones, 2003). The bICP0 protein is encoded by IE transcription unit 1 (Wirth *et al.*, 1992) and activates expression of all three classes of viral promoter (Everett, 2000). The *bICP0* gene is expressed constitutively during productive infection, as it has an IE and an E promoter that are activated by bICP0 (Figure 1a; Fraefel *et al.*, 1994). bICP0 associates with histone deacetylase 1 (HDAC1) and, in quiescent cells, bICP0 relieves HDAC1-mediated repression of transcription (Zhang & Jones, 2001). The ability of bICP0 to interact with HDAC1 may stimulate viral gene expression. Construction of a mutant BHV-1 that does not express the bICP0 protein has demonstrated that bICP0 plays an important role in productive infection of cultured bovine cells (Geiser *et al.*, 2005).

The ICP0 homologues encoded by BHV-1 and *Human herpesvirus 1* (herpes simplex virus type 1, HSV-1) contain a well-conserved C_3HC_4 zinc RING finger near their respective N termini. Mutational analysis has demonstrated the importance of the C_3HC_4 zinc RING-finger domain of bICP0 and ICP0 (Everett, 1987, 1988; Everett *et al.*, 1993; 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) co-localize with and disrupt the proto-oncogene promyelocytic leukaemia protein-containing nuclear domains (ND10 or PODs). ICP0 regulates steady-state levels of cellular and viral proteins, due to its interaction with the protein-degradation machinery (Everett *et al.*, 1997, 1999a) and E3 ubiquitin ligase activity (Boutell *et al.*, 2002; Van Sant *et al.*, 2001). The E3 ubiquitin ligase activity of ICP0 disrupts the cell cycle and alters cellular gene expression (for example, *p21*, *gadd45* and *mdm*-2) (Hobbs & DeLuca, 1999; Lomonte & Everett, 1999).

During productive infection, HSV-1 DNA is associated with histones (Herrera & Triezenberg, 2004; Kent *et al.*, 2004), suggesting that efficient viral gene expression requires an 'open'

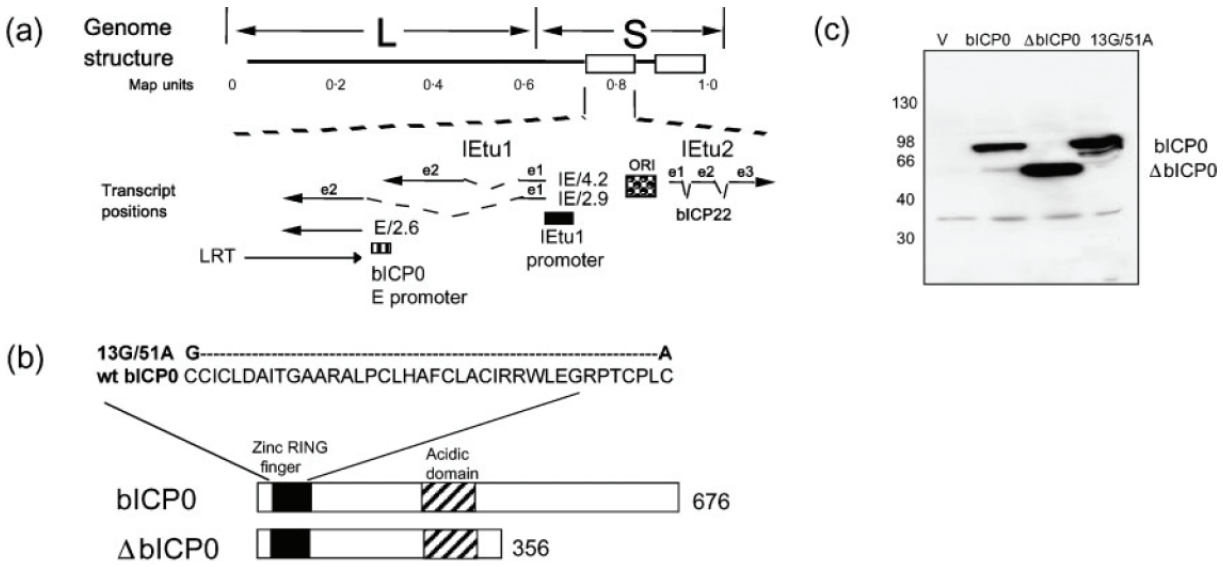

Figure 1. Schematic of the BHV-1 genome and bICP0 protein-coding sequences. (a) Schematic of BHV-1 genes in the repeats. The positions of IE transcripts (Fraefel *et al.*, 1993; Wirth *et al.*, 1989, 1991, 1992) and the LR transcript (Devireddy *et al.*, 2003; Hossain *et al.*, 1995; Kutish *et al.*, 1990) are presented. IE/4.2 encodes bICP4 and IE/2.9 encodes bICP0. One IE promoter (denoted by a filled rectangle) activates expression of IE/4.2 and IE/2.9 and this IE transcription unit is designated IEtu1. E/2.6 is the early transcript that encodes bICP0 and an early promoter (denoted by a striped box) activates expression of this transcript. Exon 2 (e2) of bICP0 contains all of the protein-coding sequences of bICP0. The origin of replication (ORI) separates IEtu1 from IEtu2. IEtu2 encodes the bICP22 protein. Solid lines in the transcript position map represent exons (e1, e2 and e3). (b) The bICP0 protein-coding sequences were cloned into a Flag-tagged CMV expression plasmid (pCMVbICP0) and this construct was designated bICP0 (Inman *et al.*, 2001b). The positions of the zinc RING finger and the acidic domain have been described previously (Wirth *et al.*, 1992). The *Sal*I site in bICP0 was used to construct the ΔC terminus construct. The simian virus 40 poly(A) addition site is located at the 3' terminus of the bICP0 insert. The amino acid sequences of theC_3HC_4 zinc RING fingers of BHV-1 bICP0 (aa 13–51) are presented. The mutations in bICP0 are shown (aa 13 was changed from C to G and aa 51 from C to A). The procedures for site-directed mutagenesis have been described previously (Inman *et al.*, 2001b). (c) 293 cells ($2x10^6$) were transfected with CMV Flag-tagged expression plasmids expressing wt bICP0, the ΔbICP0 protein, the 13G/51A mutant protein or an empty expression vector (pcDNA3.1) (V) using 20 μg DNA. At 48 h post-transfection, cell lysate was prepared and a Western blot was performed using methods described previously (Inman *et al.*, 2001b; Zhang & Jones, 2001). The Flag-tagged bICP0 proteins were detected with a Flag-specific antibody (Stratagene).

chromatin structure. When BHV-1 DNA is transfected into permissive cells, plaque formation is inefficient unless bICP0 or HSV-1 ICP0 is included in the transfection mix (Geiser & Jones, 2003; Inman *et al.*, 2001b). When the adenovirus E1A gene is co-transfected with BHV-1 DNA, plaque formation is also increased (Geiser & Jones, 2003), suggesting that E1A and bICP0 share certain common functions. The ability of the E1A protein to bind the histone acetyltransferase p300 and inhibit its histone acetyltransferase (HAT) activity promotes adenovirus productive infection (Chakravarti *et al.*, 1999; Hamamori *et al.*, 1999).

p300/CBP [cAMP response element-binding protein (CREB)-binding protein] is a ubiquitously expressed, global transcriptional co-activator that interacts with numerous DNA-binding transcription factors and nuclear-hormone receptors. p300 plays pivotal roles in many cellular processes, including cell-cycle control, differentiation and apoptosis (Chan & La Thangue, 2001; Vo & Goodman, 2001). p300 possesses intrinsic HAT activity that, in general, stimulates transcription (Grunstein, 1997; Tsukiyama & Wu, 1997) and promotes chromatin remodelling (Ogryzko *et al.*, 1996). p300 can also acetylate various transcription factors (Sterner & Berger, 2000). p300 shares several conserved functional domains: (i) the bromodomain frequently found in mammalian HATs; (ii) three cysteine/histidine-rich domains (CH1, CH2 and CH3); and (iii) a KIX domain that binds the kinase-inducible domain of CREB. The CH1, CH3 and KIX domains are important for protein–protein interactions (Eckner *et al.*, 1994; Lundblad *et al.*, 1995). Many viral proteins target p300 and these interactions induce cell-growth control, stimulate DNA synthesis and block cellular differentiation (Avantaggiati *et al.*, 1996; Eckner *et al.*, 1994; Lundblad *et al.*, 1995; Nemethova & Wintersberger, 1999; Patel *et al.*, 1999; Van Orden *et al.*, 1999; Wang *et al.*, 2000).

In this study, we have demonstrated that bICP0 associates with p300 in productively infected bovine cells or cells transfected with bIPC0. Co-transfection of p300 with BHV-1 DNA enhanced plaque formation in bovine cells. In transient-trans-

fection assays, p300 and bICP0 stimulated the BHV-1 gC promoter. When p300 and bICP0 were co-transfected with the gC promoter, gC promoter activity was higher than in cells transfected with just p300 or bICP0.

METHODS

Cells. Rabbit skin (RS) cells, Madin–Darby bovine kidney (MDBK) cells, bovine testicular cells (9.1.3) and human epithelial 293 cells were grown in Earle's modified Eagle's medium supplemented with 5 % fetal bovine serum. Fetal bovine lung (FBL) cells were maintained in Earle's modified Eagle's medium supplemented with 10 % fetal bovine serum. All medium contained penicillin (10 U ml^{-1}) and streptomycin (100 μg ml^{-1}).

Plasmids. pCMV-bICP0 contained the bICP0-coding sequences under the control of the cytomegalovirus (CMV) promoter. Mutagenesis of the bICP0 zinc RING finger has been described previously (Inman *et al.*, 2001b). The coding regions of the wild-type (wt) bICP0 and the zinc RING-finger mutant 13G/51A were inserted into the Flag-tagged expression vectors pCMV2C (bICP0) and pCMV4B (13G/51A) (Stratagene), respectively. A C-terminal deletion of bICP0 (ΔbICP0) was generated by deleting the *Sal*I–*Xho*I fragment (aa 356–676) from the Flag-tagged bICP0 construct. pCMV300 contained wild-type p300 cDNA. The dominant-negative mutation pCMV300ΔCH1 was generated by deletion of the CH1 domain (aa 348–411, nt 2242–2433). pCMV300ΔCH3 was generated by deletion of the CH3 domain (aa 1737–1836, nt 6405–6704). The p300 constructs were purchased from Upstate.

Transient expression for Western blot analysis. 293 cells (~2x10^6 in a 100 mm dish) were transfected with 20 μg of the designated bICP0 expression plasmid by using the calcium phosphate precipitation method. At 40 h post-transfection, cells were collected and lysed in 500 μl 1x SDS sample buffer [50 mM Tris/HCl (pH 6.8), 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol]. Lysed cells were boiled for 5 min and the supernatant was used for SDS-PAGE. The wt and mutant forms of the bICP0 protein were detected by using an anti-Flag antibody (Stratagene).

Co-immunoprecipitation assay. Each Flag-tagged bICP0 expression vector (10 μg) was transfected with 10 μg p300 expression vector into 293 cells (~2x10^6 in a 100 mm dish) by calcium phosphate precipitation. At 40 h post-transfection, cells were collected and suspended in 250 μl lysis buffer [20 mM HEPES/KOH (pH 7.9), 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 % glycerol, 0.5 mM dithiothreitol, complete proteinase inhibitors at one tablet per 10 ml]. Whole-cell lysate was sonicated and centrifuged for 10 min at 4 °C in an Eppendorf centrifuge (15 000 r.p.m.). The supernatant was diluted to a final volume of 1 ml with the same lysis buffer, except for the addition of 20 mM KCl. Protein G magnetic beads (25 μl) were added. The mixture was incubated at 4 °C for 1 h and the beads were collected by using a magnetic separation rack and then discarded. The supernatant was incubated with 5 μg anti-Flag antibody at 4 °C overnight. Protein G magnetic beads (25 μl) were added and incubated for 1 h at 4 °C on a rotating device. The beads were collected and washed four times with wash buffer [10 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.5 % NP-40]. After the final wash, the beads were suspended in 50 μl 2x sample buffer [100 mM Tris/HCl (pH 8.0), 20 % glycerol, 4 % SDS] and heated for 5 min at 70 °C. Detection of the precipitated protein was performed by using an anti-p300 antibody (Santa Cruz Biotechnology). Reciprocal immunoprecipitations were performed as described in Results.

Confocal microscopy. To develop an antibody against bICP0, the C terminus of bICP0 was released from pCMV2C-bICP0 by *Xho*I/*Sal*I digestion and cloned into the baculovirus expression vector pBlueBacHis2C (Invitrogen). Recombinant baculovirus was generated by using BlueBac DNA and infectious baculovirus was propagated in *Spodoptera frugiperda* 9 insect cells. The bICP0 protein was purified by nickel affinity chromatography and SDS-PAGE and the bICP0 protein was eluted from the excised band. The purified bICP0 protein was injected into rabbits to generate polyclonal antibodies. Protein G magnetic beads were used to purify the IgG. To perform confocal microscopy, MDBK cells were split into eight-well Lab-Tek culture slides and incubated for 24 h. Cultured cells were infected with BHV-1 for 4 h. After washing with PBS, infected cells were fixed in 4 % paraformaldehyde and then incubated in 100 % ethanol at –20 °C for 2 min. After washing three times with PBS, slides were blocked with 4 % BSA in PBS for 30 min and then incubated with the anti-bICP0 antibody plus the anti-p300 antibody (1 : 100 dilution; Upstate) for 2 h at room temperature. Secondary antibodies Cy2-conjugated donkey anti-rabbit IgG and Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) were added to a final concentration of 1 : 100 and incubated for 1 h at room temperature in the dark. After washing with PBS, slides were sealed with mounting gel. Images were collected by using a Bio-Rad confocal laser-scanning microscope (MRC-1024ES) with excitation/emission at 488/520 nm.

Analysing the effect of p300 on plaque formation. A BHV-1 mutant containing the β-galactosidase (β-*Gal*) gene in place of the viral gC gene was obtained from S. Chowdury (Manhatten, KS, USA) (gCblue virus). This virus grows to titres similar to those of the wild-type parental virus and expresses the β-*Gal* gene. The procedures for preparing BHV-1 genomic DNA have been described previously (Geiser *et al.*, 2002; Inman *et al.*, 2001a, b, 2002).

β-Gal activity was measured at 24 or 36 h post-transfection as described previously (Geiser *et al.*, 2002). The number of β-Gal^+ cells in cultures transfected with empty expression vector (pcDNA3.1) and viral genomic DNA was set at a value of 1 for each experiment. Following co-transfection of cultures with p300 or bICP0 and the gCblue virus, the number of β-Gal^+ cells was compared with those obtained with the empty expression vector and the gCblue virus. This representation of the data minimized the differences in cell density, Superfect lot variation and transfection efficiency.

Cloning of the BHV-1 gC promoter. The coding region of the gC gene is present in the BHV-1 *Hin*dIII-I fragment. The promoter region and the first in-frame ATG codon of the gC gene were released from the *Hin*dIII-I fragment by *Nco*I digestion. The *Nco*I fragment was treated with mung bean nuclease to remove the ATG codon. The blunt-ended fragment was cloned into the *Eco*RV site of the Flag-tagged vector pCMV2C (pCMV2C-gC). pCMV2C-gC was cut with *Eco*RI and digested with Klenow enzyme to create a blunt end. This plasmid was then digested with *Sal*I to release the gC promoter region. The reporter construct pMinCAT was digested with *Xba*I and filled in with Klenow. The TATA box of HSV-1 thymidine kinase was removed by digestion with *Xho*I. The gC promoter fragment (*Eco*RI–*Sal*I) was ligated into the *Xba*I/*Xho*I-digested pMinCAT vector (gC-CAT). To generate truncated gC promoter constructs, the upstream gC promoter sequences were removed by *Sal*I and *Xho*I digestion (gC-*Xho*I-CAT). gC-CAT was also digested with *Pst*I to remove a large portion of the promoter sequences and the plasmid was religated (gC-*Pst*I-CAT). A schematic of these constructs is shown in Figure 6(a).

Transient transfection and CAT assays. The designated gC promoter constructs and bICP0 expression plasmids were co-transfected into FBL or RS cells by using the calcium phosphate precipitation method. At 40 h post-transfection, cell lysate was prepared by three

freeze–thaw cycles in 0.25 M Tris/HCl (pH 8.0). CAT activity was measured in the presence of 0.2 μCi [^{14}C]chloramphenicol and 0.5 mM acetyl coenzyme A as described previously (Inman *et al.*, 2001b; Zhang & Jones, 2001). The various forms of chloramphenicol were separated by thin-layer chromatography and the amount of acetylated chloramphenicol was measured with a Bio-Rad Molecular Imager FX.

RESULTS

bICP0 interacts with p300 in transiently transfected cells

The rationale for testing whether bICP0 interacted with p300 was that the adenovirus E1A protein and bICP0 appear to share certain common functions (Geiser & Jones, 2003) and E1A interacts with p300 (Chakravarti *et al.*, 1999; Hamamori *et al.*, 1999). To test whether bICP0 interacted with p300, 293 cells were co-transfected with a Flag-tagged bICP0 expression construct (Figure 1b) plus a p300 expression plasmid and immunoprecipitation/Western blot analysis was performed. The bICP0 constructs used in this study, bICP0, ΔbICP0 and 13G/51A (Figure 1b), expressed similar levels of the Flag-tagged protein in transiently transfected cells (Figure 1c). When bICP0 was immunoprecipitated with the anti-Flag antibody, p300 was detected in the immunoprecipitate (Figure 2a). The interaction between p300 and bICP0 was also detected by immunoprecipitating with p300 and performing Western Blots with the anti-Flag antibody to detect bICP0 (Figure 2b). When p300 was not overexpressed, low levels of p300 were associated with bICP0 (data not shown). As these interactions were difficult to detect on a consistent basis, the results from overexpression of p300 are presented. An intact C_3HC_4 zinc RING finger was apparently not required for the interaction, as the 13G/51A protein was present in the p300 immunoprecipitate. Although the ΔbICP0 protein migrated near to the heavy chain of IgG, this truncated protein also interacted with p300.

Similar levels of p300 were detected at 40 h post-transfection when 293 cells were co-transfected with p300 and any one of the respective bICP0 constructs (Figure 2c). In agreement with a previous study (Saydam *et al.*, 2002), we were unable to detect a stable interaction between bICP0 and p53 in transiently transfected cells (Figure 2d). Furthermore, bICP0 expression did not reduce steady-state levels of p53 in transiently transfected 293 cells (Figure 2e). Previous studies have also indicated that a stable interaction is not detected between bICP0 and cyclin-dependent kinase 2 in transiently transfected cells (Zhang & Jones, 2001). In summary, the results of these studies suggested that bICP0 interacted with p300 or a p300-containing complex.

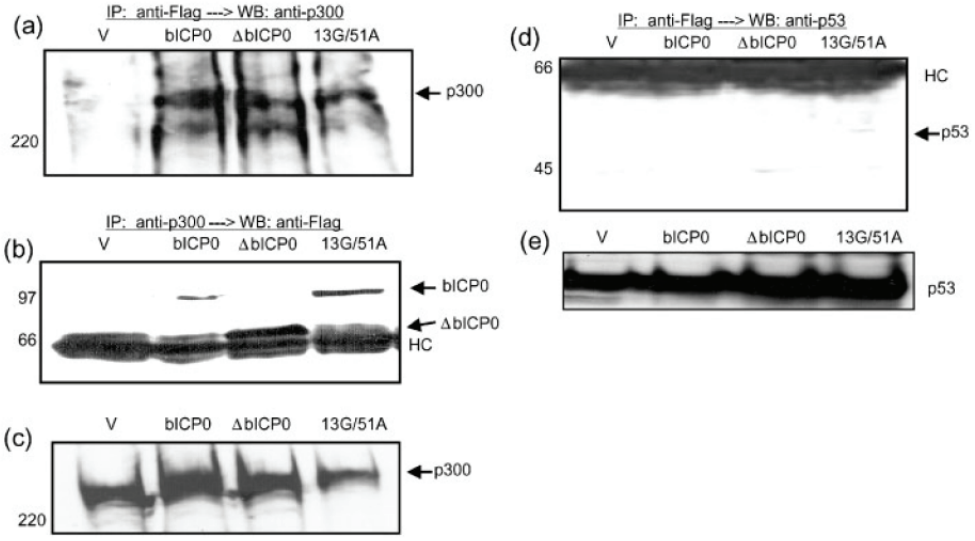

Figure 2. bICP0 interacts with p300 in transiently transfected cells. 293 cells were transfected with one of the designated bICP0 constructs (Figure 1) and p300. At 40 h post-transfection, cell lysate was prepared as described in Methods. (a) Cell lysate (1 mg) was incubated with the Flag-specific antibody to immunoprecipitate bICP0 and Western blot analysis of theimmunoprecipitate was performed by using p300-specific antibody. (b) Protein (1 mg) was immunoprecipitated by using anti-p300 antibody (Santa Cruz Biotechnology). The immunoprecipitates were separated by SDS-PAGE (10 % gel) and Western blot analysis was performed by using the Flag-specific antibody to detect the respective Flag–bICP0 fusion proteins. (c) Overall levels of p300 in transfected cells. For the Western blot, 100 μg protein was added to each lane and the proteins were separated by SDS-PAGE (6 % gel). (d) Cell lysate (1 mg) was immunoprecipitated with Flag-specific antibody and Western blot analysis of the immunoprecipitate was performed by using a p53-specific antibody (Santa Cruz Biotechnology, sc-120). (e) Total levels of p53 in transfected cells. For the Western blot, 100 μg protein was added to each lane and theproteins were separated by SDS-PAGE (10 % gel). The position of the IgG heavy chain (HC) is indicated in (b) and (d). Lane V, cell lysate prepared from cells transfected with an empty expression vector (pcDNA3.1).

bICP0 interacts with p300 in productively infected cells

Although our studies suggested that bICP0 interacted with p300 in transiently transfected cells, it was important to verify that these interactions occur in productively infected cells. To test this possibility, RS cells were transfected with 10 µg p300 expression plasmid for 12 h and then infected with BHV-1 for 24 h. RS cells were used for this study, as we could transfect at least 50 % of the cells and BHV-1 grows to high titres in RS cells. Immunoprecipitation was performed initially with a polyclonal anti-bICP0 antibody generated against the C terminus of bICP0. For these studies, an IgG fraction was used for immunoprecipitation. p300 was consistently detected in the immunoprecipitates when anti-bICP0 antiserum was used to immunoprecipitate cell lysate prepared from infected RS cells (Figure 3a). In contrast, p300 was not detected in immunoprecipitates when cell lysate prepared from mock-infected cells was immunoprecipitated with anti-bICP0 IgG. Cell lysate prepared from BHV-1-infected RS cells was also immunoprecipitated with anti-actin and anti-p300 antibodies. Anti-p300 antibody, but not anti-actin antibody, precipitated bICP0 from BHV-1-infected cells (Figure 3b). The interaction between p300 and bICP0 was also detected when p300 was not overexpressed. However, this interaction was easier to detect when p300 was overexpressed and, consequently, only these results are presented.

bICP0 does not displace p300 from the nucleus after infection

Confocal microscopy was used to test whether the association between bICP0 and p300 altered the subcellular localization of p300 after productive infection. MDBK cells were infected with BHV-1 for 4 h; cells were fixed and then immunostained with anti-bICP0 and anti-p300 antibodies. bICP0 was detected with Cy2-conjugated donkey anti-rabbit antibody (green fluorescence) and p300 was detected with Cy5-conjugated donkey anti-mouse antibody (red fluorescence). Infected cells expressing bICP0 contained p300 and bICP0 in the nucleus (Figure 4). These results demonstrated that p300 was not displaced from the nucleus after infection and confirmed that p300 levels did not decrease dramatically at 4 h after infection or later during infection (data not shown).

Analysis of the effect of p300 on plaque formation

To test whether p300 overexpression enhanced plaque formation, bovine cells were co-transfected with BHV-1 genomic DNA and increasing concentrations of p300. Transfection of bovine cells with BHV-1 genomic DNA yields low levels of infectious virus. Co-transfection of BHV-1 DNA with a plasmid expressing bICP0 enhances productive infection and virus yield (Inman *et al.*, 2001b). At 36 h post-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 resulting from virus spread. At later times post-transfection, many of the β-Gal$^+$ cells detached from the dish, making it difficult to count virus-positive cells (Geiser & Jones, 2003; Inman *et al.*, 2001b). The number of β-Gal$^+$ cells correlates directly with the number of plaques produced following transfection with the BHV-1 blue virus (Geiser & Jones, 2003; Geiser *et al.*, 2002; Inman *et al.*, 2001b). p300 consistently increased the level of plaque formation by fourfold compared with co-transfection of BHV-1 DNA with the empty expression vector (Figure 5). Relative to bICP0, p300 was less

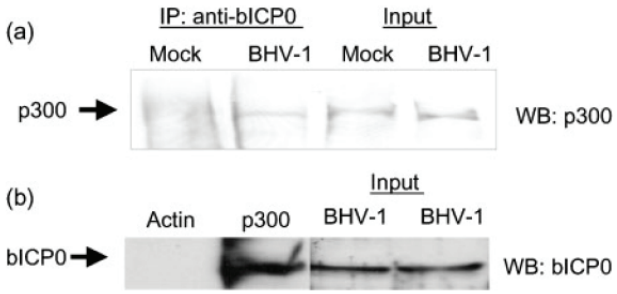

Figure 3. bICP0 interacts with p300 in productively infected cells. RS cells were transfected with 10 µg p300 expression plasmid. At 24 h post-transfection, RS cells were infected with BHV-1 for 24 h. Whole-cell lysate was then prepared as described in Methods. (a) Cell lysate (1 mg) was immunoprecipitated with 8 µg anti-bICP0 IgG antibody. Western blots were performed with anti-p300 antibody (Santa Cruz Biotechnology). (b) Whole-cell lysate (1 mg) from BHV-1-infected RS cells was immunoprecipitated with anti-actin or anti-p300 antibody, respectively. The bICP0 protein was detected with anti-bICP0 IgG. The lanes marked 'Input' contained 50 µg whole-cell lysate per lane.

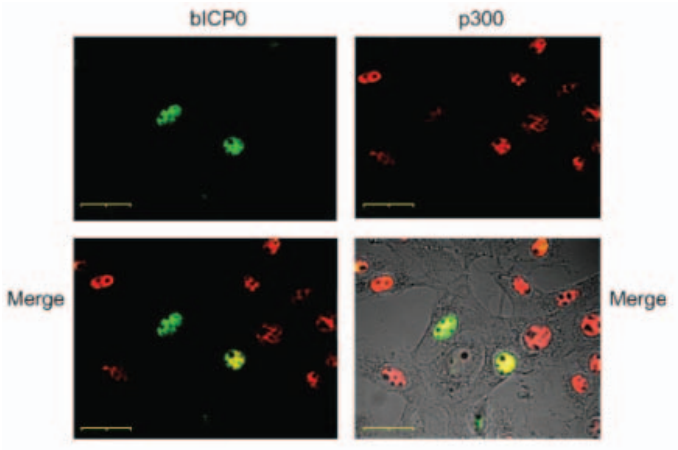

Figure 4. Nuclear localization of p300 does not change after infection. MDBK cells were infected with BHV-1 for 4 h, using an m.o.i. of 1. Cells were fixed and immunostained with rabbit anti-bICP0 and mouse anti-p300 (Upstate). bICP0 was detected by using Cy2-conjugated donkey anti-rabbit antibody and p300 was detected by using Cy5-conjugated donkey anti-mouse antibody. Bars, 20 µm.

efficient at activating productive infection; to achieve similar levels of stimulation, approximately 100-fold more p300 had to be included in the transfection mix.

Regulation of gC promoter activity by p300 and bICP0

As it is well established that p300 regulates transcription (Grunstein, 1997; Tsukiyama & Wu, 1997), we tested whether p300 had an effect on a late viral promoter (gC) that is activated by bICP0 (Wirth *et al.*, 1992). The gC promoter was cloned from the BHV-1 *Hin*dIII-I fragment and subcloned upstream of a CAT reporter gene lacking a TATA box (gC-CAT; Figure 6a). Two additional truncated promoter constructs were generated by digestion of promoter sequences with *Xho*I (gC-*Xho*I-CAT) and *Pst*I (gC-*Pst*I-CAT) (Figure 6a). The minimal gC promoter tested contained 151 bp of promoter sequences upstream of the ATG codon, a consensus TATA box and a CAAT box (Hamel & Simard, 2003). Regardless of which gC promoter construct was examined, p300 stimulated promoter activity by approximately threefold in FBL cells (Figure 6b). Deletion of the CH3 domain (ΔCH3) reduced transactivation to basal levels, whereas deletion of the CH1 domain (ΔCH1) had less of an effect.

The respective gC promoters were transactivated three- to fourfold by bICP0 in FBL cells (Figure 6b). When p300 and bICP0 were co-transfected into FBL cells, gC promoter activity increased to more than fivefold over basal levels, which was higher than bICP0 or p300 alone (Figure 6b). When the ΔCH3 construct was co-transfected with bICP0, the levels of transactivation were similar to those seen with bICP0 alone. In contrast to the results obtained with the ΔCH3 construct, the ΔCH1 construct yielded similar results to the wt p300 construct when co-transfected with bICP0. These results suggested that p300 plus bICP0 cooperate to stimulate gC promoter activity.

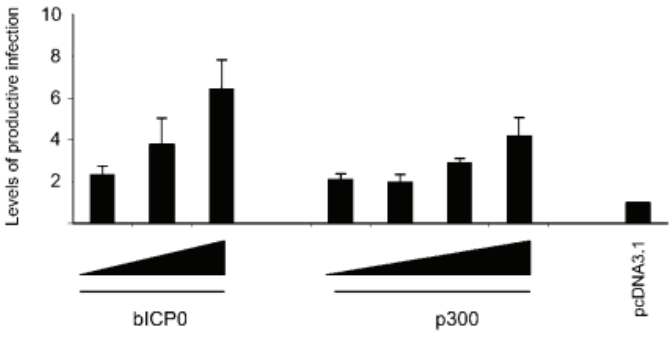

Figure 5. The *p300* gene activates productive infection. Bovine testicular cells (9.1.3) were co-transfected by using Lipofectamine 2000 with increasing amounts of plasmids expressing p300 (0.83, 1.66, 3.32 or 6.64 μg) or bICP0 (3.24, 13 or 51.9 ng) and the BHV-1 blue virus genome (0.83 μg DNA). The ratios of bICP0 molecules to viral DNA molecules were 1 : 4, 1 : 1 and 4 : 1, respectively. The ratios of p300 DNA molecules to viral genomic DNA molecules were 64 : 1, 128 : 1, 256 : 1 and 512 : 1, respectively. An empty expression vector (pcDNA3.1) was used to maintain equivalent amounts of DNA. At 24 h post-transfection, cells were fixed and stained and the number of blue cells was counted. The number of β-Gal$^+$ cells in the vector control (pcDNA3.1) was set at a value of 1 and the number of β-Gal$^+$ cells in each well was calculated as the fold of the vector control. The results are the mean ± SD of three independent experiments.

DISCUSSION

In this study, we demonstrated that bICP0 and p300 interact with each other in transiently transfected 293 cells and in

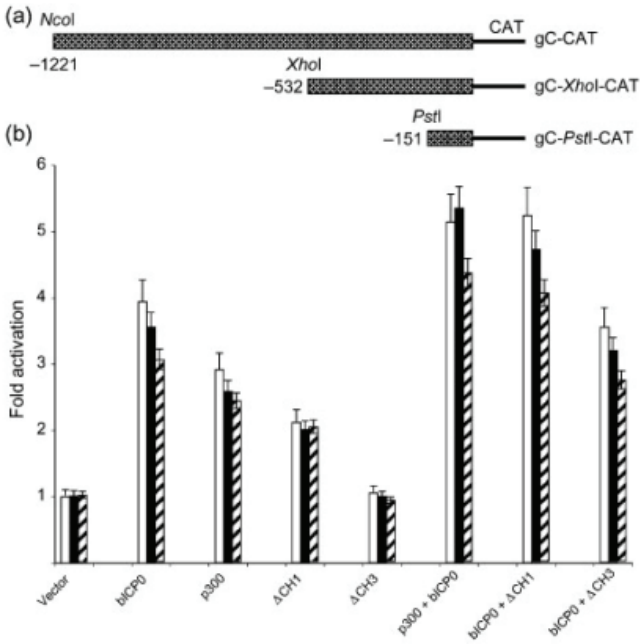

Figure 6. p300 activates the gC promoter. (a) Cloning of the gC promoter. The gC gene is located in the *Hin*dIII-I fragment. The promoter region and the first ATG codon were released by *Nco*I digestion. The ATG was removed by using mung bean nuclease and the resulting 1221 bp fragment was cloned upstream of the CAT reporter gene from vector pMinCAT, from which the TATA box had been removed. Digesting the gC promoterfragment with *Xho*I or *Pst*I generated two additional truncated promoter constructs. (b) Analysis of gC promoter activity. Each of the gC promoter constructs (16 μg) (empty bars, gC-CAT; filled bars, gC-*Xho*I-CAT; hatched bars, gC-*Pst*I-CAT), 2 μg wild-type bICP0 or 2 μg of the p300 expression vector or a mutant p300 construct was transfected into RS cells. To test whether p300 and bICP0 cooperated to activatethe gC promoter, 16 μg of one of the gC promoter constructs was co-transfected with 2 μg wild-type bICP0 and 2 μg p300 expression vector or a mutant p300 construct into RS cells. The amount of plasmid DNA for each transfection was kept the same by adding empty expression vector. At 40 h post-transfection, cell lysate was prepared and CAT activity was measured as described in Methods. These results were the mean±SD of four independent experiments.

productively infected bovine cells. In transiently transfected 293 cells, this interaction did not require an intact bICP0 zinc RING finger. The first 356 aa of bICP0, which contain the zinc RING finger, were sufficient to interact with p300 in 293 cells. Although our results suggest that bICP0 interacts with p300, they did not allow us to determine whether bICP0 interacts directly with p300 or with a p300-containing complex. Overexpression of p300 was necessary to readily detect of the interaction with bICP0 during productive infection or transfection, suggesting that other cellular proteins sequester p300 or that only a small percentage of bICP0 associates stably with p300. As many viral proteins encoded by DNA viruses interact with p300 (Vo & Goodman, 2001), we suggest that the ability of bICP0 to interact with p300 has functional significance.

Our results suggest that the interaction between bICP0 and p300 enhances productive infection (Figure 5), in part because p300 activated gC promoter activity (Figure 6). A previous study concluded that bICP0 did not transactivate the gC promoter unless the fragment encompassed nt –1155 to +71 of the gC promoter (Hamel & Simard, 2003). In the bovine cells that we tested, the minimal gC promoter (gC-*Pst*I-CAT) was transactivated by bICP0 with only slightly reduced efficiency compared with the two larger gC promoter constructs. These findings are consistent with previous findings that bICP0 is capable of activating a simple promoter if it contains a TATA box (Inman *et al.*, 2001b; Zhang & Jones, 2001; Zhang *et al.*, 2005). Additional viral promoters may be activated by p300, as the minimal gC promoter tested (gC-*Pst*I-CAT) was activated by p300. p300 and bICP0 cooperated to transactivate the gC promoter more efficiently than bICP0 or p300 alone. The cooperative effect of p300 and bICP0 on gC promoter activity may be underestimated, due to endogenous p300 that is present in cells.

p300 activates the innate immune response, in particular transcriptional activation of interferon (IFN)-dependent transcription (Munshi *et al.*, 2001; Ogryzko *et al.*, 1996; Sterner & Berger, 2000; Vo & Goodman, 2001; Weaver *et al.*, 1998; Yoneyama *et al.*, 1998). bICP0 inhibits the IFN-β promoter and a simple promoter that contains consensus IFN-stimulated response elements in several cell types (Henderson *et al.*, 2005). In addition to p300 and bICP0 cooperation to activate gC promoter activity, the interaction between bICP0 and p300 may inhibit IFN transcriptional signalling following productive infection. p300 also stimulates other antiviral signalling pathways and, as such, is a common target for viral proteins that regulate transcription and innate immunity (Vo & Goodman, 2001). For example, p300 binds to the activation domain of p53 and enhances p53-dependent transcription (Gu & Roeder, 1997; Lill *et al.*, 1997) by acetylating p53 on specific residues (Gu & Roeder, 1997; Gu *et al.*, 1997). We were unable to transactivate a simple promoter with bICP0 when consensus p53-binding sites were cloned upstream of the TATA box (Y. Zhang, unpublished data). Thus, the ability of bICP0 to interact with p300 may prevent p300 from activating antiviral signalling pathways during the course of productive infection.

In summary, we suggest that interactions between bICP0 and p300 cooperate to enhance viral gene expression and may interfere with antiviral signalling pathways (for example, IFN and perhaps the p53 pathway). Our studies also suggest that interactions between bICP0 and p300 might alter the HAT activity of p300, which could stimulate productive infection indirectly. Several lines of evidence imply that chromatin-remodelling enzymes are necessary for efficient BHV-1 productive infection: (i) bICP0 interacts with HDAC1 and inhibits the ability of HDAC1 to repress transcription (Zhang & Jones, 2001); (ii) E2F4 (a cellular protein) stimulates BHV-1 plaque formation (Geiser & Jones, 2003) and E2F4 binds HDAC family members (Trimarchi & Lees, 2002); (iii) HDAC inhibitors accelerate viral gene expression of an HSV-1 ICP0 mutant (Poon *et al.*, 2003); (iv) HSV-1 genomes in productively infected cells (Herrera & Triezenberg, 2004; Kent *et al.*, 2004) and latently infected neurons (Deshmane & Fraser, 1989) are associated with histones; and (v) BHV-1 DNA does not induce plaque formation efficiently unless bICP0 or HSV-1 ICP0 is included in the transfection mix (Geiser & Jones, 2003; Inman *et al.*, 2001a, b). Future studies will focus on understanding whether interactions between bICP0 and chromatin-remodelling enzymes (HDAC1 and p300) influence the association of histones with the BHV-1 genome.

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