The zinc ring finger in the bICP0 protein encoded by bovine herpesvirus-1 mediates toxicity and activates productive infection

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The bICP0 protein encoded by bovine herpesvirus 1 (BHV-1) is believed to activate transcription and consequently productive infection. Expression of full-length bICP0 protein is toxic in transiently transfected mouse neuroblastoma cells (neuro-2A) in the absence of other viral genes. However, bICP0 does not appear to directly induce apoptosis. Although bICP0 is believed to be functionally similar to the herpes simplex virus type 1-encoded ICP0, the only protein domain that is well conserved is a C3HC4 zinc ring finger located near the N terminus of both proteins. Site-specific mutagenesis of the zinc ring finger of bICP0 demonstrated that it was important for inducing aggregated chromatin structures in transfected cells and toxicity. The zinc ring finger was also required for stimulating productive infection in bovine cells and for trans-activating the thymidine kinase (TK) promoter of herpes simplex virus type 1. Deletion of amino acids spanning 356–677 of bICP0 altered subcellular localization of bICP0 and prevented trans-activation of the TK promoter. However, this deletion did not prevent trans-activation of the viral genome. Taken together, these studies indicated that bICP0 has several functional domains, including the zinc ring finger, which stimulate productive infection and influence cell survival.

Introduction

Bovine herpesvirus (BHV)-1 infection can cause conjunctivitis, pneumonia, genital disorders, abortions, occasionally encephalitis, and a complex upper respiratory infection referred to as ‘shipping fever’. BHV-1 is not the sole infectious agent associated with shipping fever; however, infection initiates the disorder by immunosuppressing infected cattle (Tikoo et al., 1995). Consequently, secondary bacterial infections by Pasteurella haemolytica, Pasteurella multocida and Haemophilus somnus can cause pneumonia. Although vaccines are available, they can cause disease in calves or abortions in cows. Generation of effective vaccines is further complicated by the fact that BHV-1 establishes life-long latent infections in sensory ganglionic neurons (Jones, 1998).

Infection of permissive cells with BHV-1 leads to rapid cell death, in part because of apoptosis (Devireddy & Jones, 1999). Viral gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E) or late (L) (Jones, 1998).

IE gene expression is stimulated by a virion component, bTIF. bTIF interacts with a cellular transcription factor (Oct-1) and transactivates IE gene expression (Misra et al., 1995, 1996). Two IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2 (Wirth et al., 1989, 1991, 1992). The proteins encoded by IEtu1 are believed to be functional homologues of herpes simplex virus (HSV)-1 proteins ICP0 and ICP4. IEtu2 encodes a protein that is similar to a non-essential HSV-1 IE gene, ICP22 (Schwyzer et al., 1994). bICP0 is translated from an IE (IE2.9) or E mRNA (E2.6) and is considered the major viral regulatory protein. bICP0 activates its own expression as well as other viral genes and is thus considered to be a ‘promiscuous transactivator’ (IE, E and L transcription units) (Wirth et al., 1992; Koppel et al., 1997). Two independent studies concluded that bICP0 is toxic to mammalian cells (Ciacci-Zanella et al., 1999; Steinmann et al., 1998). Taken together, these studies suggested that bICP0 has several functions during productive infection.

bICP0 is believed to be a functional homologue of the HSV-1 ICP0 gene, despite having only limited sequence similarity. The only well-defined domain that is conserved is a C3HC4 zinc ring finger located near the N terminus of both
proteins (Everett, 1987, 1988, 2000; Everett et al., 1993a, b, 1995; Lium & Silverstein, 1997). Disruption of the HSV-1 zinc ring finger has profound effects on HSV-1 growth. The N terminus of HSV-1 ICP0 is also required for IE promoter activation; however, a separate domain activates E or L promoters (Lium et al., 1998; Lium & Silverstein, 1997). Thus, ICP0 can activate expression of all viral genes, in large part because it increases steady state levels of mRNA (Jordan & Schaffer, 1997). ICP0 binds several cellular proteins: (1) a translational elongation factor 1a (Kawaguchi et al., 1997a), (2) a C-terminal portion of the viral genome and the HSV-1 thymidine kinase promoter, indicating that the ring finger is important for trans-activation of the viral genome and the HSV-1 thymidine kinase promoter, indicating that the ring finger is important for the biological properties of bICP0.

In this study, we examined the effect that bICP0 has on toxicity, productive infection and gene expression. bICP0 does not directly lead to apoptosis in transiently transfected cells. However, cells transfected with bICP0 contain unusual chromatin structures, which are not frequently detected when site-specific mutations are introduced in the bICP0 zinc ring finger. The organization of chromatin appeared to be similar to that of cells arrested in the G2/M phase of the cell cycle. A C-terminal deletion mutant of bICP0 was also toxic to transfected cells, suggesting that sequences within the N terminus of bICP0 mediate toxicity. The intact zinc ring finger was necessary for trans-activation of the viral genome and the HSV-1 thymidine kinase promoter, indicating that the ring finger is important for the biological properties of bICP0.

**Methods**

### Cells and viruses

Cells were plated at a density of $5 \times 10^5$ per 100 mm² plastic dish in Earle’s modified Eagle’s medium supplemented with 5% foetal bovine serum (FBS), penicillin (10 U/ml) and streptomycin (100 µg/ml). Bovine kidney (MDBK) (ATCC CCL-22) and mouse neuroblastoma (neuro-2A; ATCC CCL131) cells were split at a 1:5 ratio every 4–5 days. Neuro-2A cells exhibit certain neuronal features (Devireddy & Jones, 2000) but can be readily grown in tissue culture.

The other bovine cell line used in this study was generated by transfecting primary bovine skin cells with the simian virus 40 Large T-antigen gene (Hegde et al., 1998). One of the immortalized cell lines was designated 9.1.3 and these cells have fibroblast-like characteristics.

A BHV-1 strain containing the β-Gal gene in place of the viral gC gene was kindly donated by S. Chowdury (Manhattan, KS, USA) (BHV-1 blue virus). This virus grows to similar titres as the wild-type parent virus and expresses β-Gal as a late gene because it is regulated by the gC promoter.

### Extraction of viral DNA

MDBK cultures were infected with an m.o.i. of 10 for 1 h, the cultures subsequently rinsed twice with calcium/magnesium-free PBS (CMF-PBS), and new media added. At 16–36 h post-infection (CPE was approximately 80%), the supernatant was collected and clarified by centrifugation (7,000 r.p.m., 4 °C, 20 min in a Beckman J2-21 using a JA-20 rotor). Virus was subsequently pelleted using a 30% sucrose/PBS cushion (25 ml virus/5 ml sucrose solution) by centrifugation (25,000 r.p.m. for 2 h in a Beckman L7-85 ultracentrifuge using an SW28 rotor at 4 °C). The pellet was suspended in 1–8 ml of DNase I-free TE. The virions were disrupted by adding 100 µl 10% SDS, 15 µl 1 mg/ml RNase, and incubating the solution at 37 °C for 30 min. Proteinase K (100 µl of a 10 mg/ml solution) was added and the solution was incubated at 56 °C for 30 min. Three phenol–CHCl₃–isoamyl alcohol (50:48:2) extractions were performed, followed by one extraction with CHCl₃–isoamyl alcohol (48:2:1). The resulting aqueous layer was subjected to three extractions with ether. DNA was precipitated with 2.5 vols 95% ethanol and sodium acetate (0.3 M). Viral DNA was electrophoresed on an agarose gel to examine its quality and quantity. Known concentrations of DNA standards were used to accurately estimate the amounts of viral DNA.

### Transfections

Cells were cotransfected with the indicated plasmids or viral genomic DNA by either calcium phosphate precipitation (Caci-Zanella et al., 1999) or Superfect (Qiagen). Superfect transfection was performed as per the manufacturer’s instructions. Briefly, 1 h prior to transfection, 1 × 10⁶ cells per well were plated into six-well plates. The designated plasmids and genomic DNA were used for transfection. The total volume was adjusted to 100 µl by adding medium without serum or antibiotics followed by 12–15 µl of Superfect. The solution was incubated at room temperature for 10 min, and the cells were rinsed with CMF-PBS; 600 µl of medium with serum and antibiotics was then added to DNA/Superfect and this solution added to cells. After 3 h at 37 °C, cultures were rinsed three times and fresh medium was added.

### DNA staining

Hoechst 33342 staining was performed as previously described (Devireddy & Jones, 1999). For these studies, cells were grown in six-well plates.

### Mutagenesis of the bICP0 zinc ring finger

pCMV-bICP0 contains the bICP0 coding sequences under control of the human CMV promoter and was kindly provided by M. Schwyzer (Zürich, Switzerland) (Fig. 1). The HindIII–ApaI fragment (308 bp) of bICP0 contains the N-terminal portions of bICP0 coding sequences and was released from pCMV-bICP0. This 308 bp fragment was cloned into pGEM-11zf (pGEM-bICP0) (Promega). To generate 13G/51A double mutants of the C₁₂H₁₂ zinc finger domain, two rounds of sequential PCR were performed (Ausubel, 1995) using two sets of primers. The sequence of the primers is as follows.

1. **13G+** S’ GCTGGGATCCGCTGATCCTTG 3’
2. **13G−** G’ CAGATGCAAGCCATCCCAGC 3’
3. **51A+** GACCTGCCCCTGGCAAGCCCGCCGTG 3’
4. **51A−** CACGGGCGCTTGGCCAGCGGGCAGGTC 3’

The flanking primers were M13 forward and M13 reverse respectively.

Primary PCR was carried out in a total volume of 20 µl containing 100 ng of pGEM-bICP0, 50 nM mutagenic primer and flanking primer respectively, 250 µM 4 × dNTP, 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 5% glycerol and 2.5 µl of Taq DNA polymerase. The PCR was performed as follows: 94 °C for 2 min; 25 cycles consisting of 94 °C for 45 s, 45 °C for 2 min, 72 °C for 1 min; final extension at 72 °C for 5 min. Klenow fragment (5 µl) was added to the reaction mix and incubated for 15 min at 30 °C. The PCR products were purified from 2% agarose gel and used as a template for secondary PCR. The secondary PCR was performed as above with the following modifications: 50 ng of the first PCR product, 500 nM flanking primers and annealing tem-
per temperature changed to 50 °C. Amplified fragments were purified from a 2% agarose gel, digested with HindIII and ApaI, and cloned into pGEM-11zf. DNA sequencing was performed to confirm that the mutations were present. The mutated HindIII–ApaI fragment was introduced into the same sites of pCMV-bICP0 construct. The resulting mutant had two changes in conserved amino acids of the C-terminal zinc ring finger (Fig. 1C) and the construct was designated mblCP0.

The HindIII–XhoI fragment (2548 bp) from plasmid pCMV-bICP0 (contains the entire protein coding sequences of bICP0) was inserted into a Flag-tagged expression vector, pCMV-2C (pCMV2-bICP0) (Stratagene). The PstI fragment, nucleotides 327–2327, containing the bICP0 coding sequences (2 kb DNA) from plasmid pCMV-13G/51A (mblCP0), was inserted into a Flag-tagged expression vector, pCMV-4B (pCMV4-bICP0) to construct a C-terminal deletion of bICP0 that includes amino acids 356–676, a SalI–XhoI fragment (1466 bp) from plasmid pCMV2-bICP0 was deleted (AC-terminus). A similar construct containing deletion of the C-terminal 320 amino acids and disruption of the zinc ring finger was also constructed (AC-Zinc finger). AC-Zinc finger was constructed by digesting mblCP0 with SalI, which released the C-terminal portion of bICP0. The digested plasmid was then ligated.

### Transient Expression and Western blot analysis.

Human epithelial cells (293) (ATCC CRL 1573) were transfected with 20 μg of the respective expression plasmids by calcium phosphate precipitation (Zhang et al., 1999); 48 h after transfection, cells were collected and lysed in 500 μl of 1× SDS sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). The cell extract was boiled for 5 min and the supernatant used for SDS–PAGE. Immunodetection of bICP0 or mbICP0 was performed as described previously (Ciacci-Zanella et al., 1998) with a monoclonal anti-Flag antibody (Stratagene).

For measuring chloramphenicol acetyltransferase (CAT) enzymatic activity, 15 μg of pMinCAT and 6 μg of the designated bICP0 constructs were cotransfected into CV-1 cells. Plasmid pMinCAT contains a portion of the HSV-1 thymidine kinase (TK) promoter (−32 to +51) fused to the chloramphenicol acetyltransferase (CAT) gene (Kretzner et al., 1992) and was obtained from L. Kretzner (University of South Dakota, USA). At 24 h after transfection, cells were lysed and CAT assays performed (Delhorn & Jones, 1997; Devireddy & Jones, 1999; Zhang et al., 1999).

### bICP0 localization.

Confocal microscopy was used to examine the subcellular localization of bICP0. For these studies, the Flag-tagged bICP0 constructs were used. Neuro-2A cells were transfected by calcium phosphate precipitation using 8 μg of the designated Flag-tagged bICP0 constructs or the empty expression vector (pCMV2C). After 16 h, the cultures were split into eight-well Lab-Tek slides with a cell density of 0.5 × 10^5 per chamber. After incubating for 48 or 72 h, the cultures were rinsed with medium that did not contain serum and then with PBS. Slides were then incubated at −20 °C for 5 min with cold 100% ethanol after which the cells were washed twice with PBS. Blocking was performed for
30 min with 4% BSA in PBS. Samples were then incubated with the Flag antibody (1:100) for 1 h at 20°C and rinsed three times with PBS containing 0.05% Tween 20. The secondary antibody, FITC goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (1:100) was added and incubated for 1 h at 20°C in the dark. Slides were then washed twice with PBS containing 0.05% Tween 20 and once with PBS. After a coverslip was added, the images were collected using a Bio-Rad confocal laser-scanning microscope (MRC-1024ES) with excitation/emission at 488/520 nm.

Results

Site-directed mutagenesis of the zinc ring finger

For the studies described below, a plasmid containing the entire coding sequences of bICP0 was used (pCMV-bICP0; Fig. 1B). The zinc ring finger in bICP0 was mutated by changing two amino acids (Fig. 1C) and this construct designated mbICP0. The amino acids that were mutagenized in the zinc ring finger of bICP0 are conserved between HSV-1 and BHV-1 (Fig. 1C). Mutation of these conserved cysteine residues in the HSV-1 ICP0 ring finger has a profound effect on virus growth (Everett et al., 1993a, b, 1995; Everett, 1987, 1988, 2000; Liou & Silverstein, 1997). mbICP0 (Fig. 2, lane 3) and wild-type bICP0 (lane 2) migrated near 100 kDa when expressed as a Flag-tagged fusion. Deletion of 320 amino acids (position 356–676) at the C terminus (ΔC-terminus) resulted in synthesis of a truncated protein migrating with an apparent molecular mass of approximately 55 kDa (lane 3). Relative to bICP0, higher levels of mbICP0 and the ΔC-terminus protein were consistently detected when equivalent amounts of cell lysate were analysed.

The subcellular localization of the wild-type bICP0 protein and the mutant proteins was examined in transiently transfected neuro-2A cells using a monoclonal antibody directed against the Flag epitope. At 48 h after transfection, many neuro-2A cells transfected with bICP0 contained Flag staining primarily in the cytoplasm, but little staining in the nucleus (Fig. 3). At 72 h after transfection, neuro-2A cells transfected with the wild-type construct contained bICP0 that was primarily expressed in the nucleus. Nuclear staining was seen in cells transfected with the mbICP0 construct at 48 or 72 h after transfection. Although most cells transfected with bICP0 or mbICP0 had exhibited nuclear staining at 72 h after transfection, cells transfected with mbICP0 appeared to be distinct. The punctate nuclear staining of bICP0 is consistent with a recent report which concluded that bICP0 colocalizes with nuclear structures (Parkinson & Everett, 2000). Flag staining was observed in the cytoplasm and nucleus when neuro-2A cells were transfected with ΔC-terminus, regardless of the time after transfection. Many neuro-2A cells transfected with the ΔC-terminus construct contained projections that were Flag-positive. Neuro-2A cells transfected with bICP0 or mbICP0 did not typically have these projections. In summary, the subcellular localization of bICP0 in neuro-2A cells was mediated by sequences in the last 320 amino acids and the zinc ring finger.

Analysis of the toxic effect of bICP0

HSV-1-encoded ICP0 localizes with nuclear structures during acute infection, which ultimately leads to their disruption (Everett et al., 1991, 1993b, 1995, 1999b; Everett, 1988, 2000). It also appears that bICP0 interacts with nuclear structures (Fig. 3, and Parkinson & Everett, 2000). Apoptotic promoting proteins can alter cell structure and translocate cellular proteins (White, 1996). Previous studies provided evidence that bICP0 had a toxic effect on transiently transfected cells (Ciacci-Zanella et al., 1999), suggesting that bICP0 induced apoptosis. Consequently, we compared the toxic effects of bICP0 to the zinc ring finger mutant and determined whether apoptosis was the mechanism for toxicity.

Initial experiments examined DNA content of transfected cells and fragmentation of DNA. When neuro-2A cells were transfected with bICP0, subgenomic apoptotic peaks were not detected by flow cytometry and DNA ladders were not readily apparent on agarose gels (data not shown). Neuro-2A cells are murine neuroblastoma cells that are readily transfected and sensitive to chemicals that induce apoptosis (Ciacci-Zanella et al., 1999; Perng et al., 2000). Hoechst 33342 was also used to visualize cellular DNA in neuro-2A cells transfected with bICP0. As a comparison, neuro-2A cells were treated with sodium butyrate, a pro-apoptotic agent that readily kills neuro-
Fig. 3. Subcellular localization of bICP0 in transiently transfected cells. Neuro-2A cells were transfected with the designated plasmids. At the indicated times after transfection, the cells were fixed and processed for confocal microscopy as described in Methods. As a negative control, cells were transfected for 48 h with a blank Flag expression vector (pCMV2C).
Fig. 4. Analysis of bICP0-transfected cells. Neuro-2A cells were cotransfected with plasmid pCMV-β-gal (human CMV β-Gal expression plasmid; Invitrogen) (2 µg) and an empty vector control or the designated bICP0 constructs (4 µg) using the calcium phosphate method. Forty-eight hours after transfection, cells were fixed and incubated with Hoechst 33342 to stain nucleus DNA. The arrows point to cells that were β-Gal\(^+\). Cultures were also treated with 5 mM sodium butyrate for 24 h and then stained with Hoechst 33342. The morphological changes in the cells that were treated with sodium butyrate are consistent with cells undergoing apoptosis (denoted by arrows).

2A cells (Ciacci-Zanella et al., 1999). Since we cotransfected the cells with pCMV-β-Gal and a bICP0 construct, we could identify β-Gal\(^+\) cells and then observe cellular chromatin in cells by fluorescent microscopy. Cells treated with sodium butyrate contained condensed chromatin, which was not frequently observed in β-Gal\(^+\) cells transfected with pCMV2C (Fig. 4). Cells cotransfected with bICP0 and pCMV/β-gal contained β-Gal\(^+\) cells with aggregated chromatin having cross-like configurations. The chromatin organization appeared to be similar to that of cells arrested in the G\(_2\) phase of the cell cycle. Neuro-2A cells transfected with mbICP0 did not contain β-Gal\(^+\) cells with aggregated chromatin or cross-like chromatin.

Following cotransfection of neuro-2A cells with a plasmid containing the β-Gal gene and one of the following bICP0 constructs, the number of β-Gal\(^+\) cells was counted. At 55–65 h after transfection the cultures were assayed for β-Gal expression. This assay allows quantification of cells that undergo apoptosis or those that are killed by non-apoptotic mechanisms (Ciacci-Zanella et al., 1999; Ciacci-Zanella & Jones, 1999; Hsu et al., 1995; Perng et al., 2000). Neuro-2A cells transfected with
Zinc ring finger of bICP0 mediates activity

bICP0 or ΔC-terminus contained at least 50% fewer β-Gal+ cells (Fig. 5). In contrast, mbICP0 had similar numbers of β-Gal+ cells as cultures transfected with the vector control. The ΔC-Zinc finger construct was more toxic than mbICP0 but appeared to be less toxic than ΔC-terminus. In summary, these studies indicated that wild-type bICP0 altered the chromatin structure of neuro-2A cells and was toxic to cells, but did not directly induce apoptosis.

The zinc ring finger is important for productive infection and TK promoter activity

To test whether the zinc ring finger and the C terminus stimulated productive infection, we cotransfected an immortalized bovine skin cell line (9.1.3) with the viral genome and one of the bICP0 constructs. For this study, we used a BHV-1 strain that contains the β-Gal gene inserted downstream of the gC promoter in place of the gC ORF (BHV-1 blue virus). β-Gal expression directly correlates with virus replication because the gC promoter is a late promoter and its expression is low prior to viral DNA replication. We used 24 h after transfection as the time to measure β-Gal+ cells to minimize counting virus-positive cells that resulted from virus spread. At later time-points, bICP0 caused β-Gal+ cells to lift off the dish making it more difficult to accurately count the virus-positive cells. bICP0 increased the number of β-Gal+ cells more than 4-fold at 24 h after transfection when a viral DNA to bICP0 ratio of 64:1 was used (Fig. 6). A 64:1 ratio (µg BHV-1 DNA to µg bICP0 plasmid DNA) is approximately a 1:1 ratio based on the number of molecules. In contrast, there was no stimulation when a ratio of 1:1 (viral DNA to bICP0 DNA) was used (data not shown), perhaps as a result of bICP0 toxicity. mbICP0 and the ΔC-Zinc finger construct only increased the number of β-Gal+ cells 2-fold. Surprisingly, the ΔC-terminus construct increased the number of β-Gal+ cells nearly as efficiently as bICP0. The number of β-Gal+ cells correlated with the efficiency of plaque formation and virus production (data not shown), suggesting that this assay can be used to test the effect of various genes on productive infection.

To determine whether the inability of mbICP0 to activate productive infection correlated with activation of transcription, we tested the ability of the bICP0 constructs to stimulate the HSV-1 TK promoter in transiently transfected cells. The TK promoter construct used for this study contains sequences spanning −32 to +51 (Fig. 7A). bICP0 activates pMinCAT (TK promoter construct) in transiently transfected cells (Bratanich et al., 1992, and Fig. 7B). mbICP0 and ΔC-terminus were unable to activate the TK promoter when compared to the blank expression vector (pCMV2C). In summary, these studies demonstrated that an intact zinc ring finger was necessary for activation of the viral genome and trans-activation of the TK promoter. Deletion of the C-terminal 320
Discussion

All alphaherpesvirus that have been sequenced contain a protein related to HSV-1 ICP0 (Everett, 2000; Lium et al., 1998; Lium & Silverstein, 1997). Each of these ICP0 homologues contains a conserved C<sub>2</sub>H<sub>2</sub>C<sub>2</sub> zinc ring finger near their respective N terminus and stimulates viral gene expression. The N terminus of HSV-1 ICP0 (amino acids 1–103) contains a transcriptional activation domain that stimulates IE promoter activity (Lium et al., 1998; Lium & Silverstein, 1997). C<sub>2</sub>H<sub>2</sub>C<sub>2</sub> zinc ring fingers are also found in several cellular proteins that regulate transcription, differentiation and oncogenesis (Freemont et al., 1991; Lovering et al., 1993). The mutated amino acids in bICP0 fall within the well-conserved C<sub>2</sub>H<sub>2</sub>C<sub>2</sub> zinc ring finger. Mutagenesis of the analogous amino acids in the HSV-1 ICP0 protein has deleterious effects (Lium & Silverstein, 1997). Although the BHV-1 and HSV-1 ICP0 proteins are considered to be functional homologues, the two proteins may have significant differences because the remainder of the protein is not well conserved. Furthermore, transcriptional regulation of bICP0 is distinct relative to HSV-1 ICP0 (Wirth et al., 1992). For example, two distinct promoters, an IE and E promoter, regulate bICP0 expression. The IE promoter of bICP0 also drives bICP4 expression and the bICP4 protein represses this IE promoter. Since bICP0 activates its own E promoter, bICP0 is hypothesized to be the major regulatory protein of BHV-1.

Deletion of the C-terminal domain and disruption of the zinc ring finger altered the subcellular localization of bICP0. It was predicted that bICP0 would be present in the nucleus of transfected cells because of its potential role in transcription and thus its detection in the cytoplasm was surprising. However, HSV-1 ICP0 can be detected in the nucleus and cytoplasm of productively infected cells and in transiently transfected cells (Everett et al., 1991, 1993a, b, 1995; Everett, 1987, 1988, 2000). As with bICP0, sequences at the C terminus of HSV-1 ICP0 regulate its subcellular localization. Furthermore, ICP27 inhibits localization of ICP0 to the nucleus whereas ICP4 promotes nuclear localization (Zhu et al., 1994). An arenavirus zinc ring finger protein binds the oncoprotein promyelocyte leukaemia protein and relocates nuclear bodies to the cytoplasm (Borden et al., 1998). The ability of zinc ring finger proteins, including bICP0, to localize in the nucleus and cytoplasm is likely to have functional significance.

Although bICP0 is toxic, it does not appear to directly induce apoptosis in transiently transfected neuro-2A cells. This conclusion is supported by the finding that transfected cells lacked condensed chromatin and increased amounts of cleaved genomic DNA. Both of these changes are hallmarks of cells undergoing apoptosis. Furthermore, cellular anti-apoptotic genes were unable to reduce the toxicity of neuro-2A cells transfected with bICP0 (data not shown). The zinc ring finger of bICP0 played an important role in toxicity because cells transfected with mbICP0 did not contain unusual chromatin structures and appeared to grow normally. In the context of the viral genome, bICP0 may indirectly promote apoptosis in cultured cells (Devireddy & Jones, 1999) and cattle (Winkler et al., 1999) by stimulating productive infection.

In contrast to HSV-1 DNA, BHV-1 DNA is not very infectious unless cotransfected with bICP0 (Fig. 6) or HSV-1 ICP0 (M. Inman & C. Jones, unpublished). The BHV-1 IE promoter that regulates bICP0 and bICP4 is dependent on bTIF expression (Misra et al., 1995), suggesting that productive infection does not proceed efficiently in the absence of bTIF or other factors that induce bICP0 expression. The inability of mbICP0 to stimulate productive infection correlated with its inability to trans-activate the HSV-1 TK promoter. The C-terminus construct activated productive infection but was unable to activate TK promoter activity in transiently transfected cells. This suggested that bICP0 has more than one functional domain or that sequences at the N terminus of
bICP0 activated expression of bICP0 in the gC virus. The finding that the N terminus of HSV-1 ICP0 is required for activating IE promoters but is not necessary for activating E or L promoters (Lium et al., 1998) supports the hypothesis that ICP0 proteins are multi-functional. A bICP0 mutant was constructed in which β-Gal-coding sequences were inserted into the bICP0 locus (Koppel et al., 1996). Although it is clear that this mutant yields 2–3 logs less than wild-type virus, its role in pathogenesis has not been tested. Thus, construction of additional bICP0 mutants will be useful for understanding the role of bICP0 in latency and pathogenesis.

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