Identification of functional domains within the bICP0 protein encoded by bovine herpesvirus 1

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It is believed that the bICP0 protein encoded by bovine herpesvirus 1 (BoHV-1) stimulates productive infection by activating viral gene expression. Like the other ICP0-like proteins encoded by alphaherpesvirinae subfamily members, bICP0 contains a zinc RING finger near its amino terminus. The zinc RING finger of bICP0 activates viral transcription, stimulates productive infection, and is toxic to certain cell types. Apart from the zinc RING finger, bICP0 possesses little similarity to the herpes simplex virus type 1 ICP0 protein making it difficult to predict what regions of bICP0 are important. To begin to identify bICP0 functional domains that are not part of the zinc RING finger, a panel of transposon insertion mutants that span bICP0 was developed. A large domain spanning aa 78–256, and a separate domain that is at or near aa 457 was necessary for efficient transactivation of a simple promoter. Transposon insertion at aa 91 impaired bICP0 protein stability in transfected cells. Insertion of transposons into the acidic domain of bICP0 had little or no effect on transactivation of a simple promoter or protein expression suggesting this region does not play a major role in activating gene expression. Sequences near the C terminus (aa 607–676) contain a functional nuclear localization signal. Collectively, these studies indicated that bICP0 contains several important functional domains: (i) the zinc RING finger, (ii) two separate domains that activate transcription, and (iii) a C-terminal nuclear localization signal that is also necessary for efficient transactivation.

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is an alphaherpesvirinae subfamily member, and it shares a number of biological properties with herpes simplex virus type 1 (HSV-1) or HSV-2. BoHV-1 infection can cause conjunctivitis, pneumonia, genital disorders, abortions, occasionally encephalitis, and a complex upper respiratory infection referred to as ‘shipping fever’. BoHV-1 establishes lifelong latent infections in sensory ganglionic neurons, and can be reactivated periodically upon stress or immunosuppression (reviewed by Jones, 1998, 2003). During productive infection, BoHV-1 gene expression occurs in three phases: immediate-early (IE), early (E) or late (L). IE transcripts can be expressed in the absence of protein synthesis, and are stimulated by a virion component, bTIF (Misra et al., 1994, 1995). bTIF interacts with a cellular transcription factor (Oct-1) and transactivates IE gene expression. Three proteins, bICP0, bICP4 and bICP22, are encoded by the IE genes of BoHV-1. bICP0 is expressed from an IE (IE2.9) or E mRNA (E2.6), and the bICP0 protein activates both promoters (Wirth et al., 1992).

The ICP0 homologues encoded by BoHV-1 and HSV-1 contain a well-conserved C3HC4 zinc RING finger near their respective N termini. Mutational analysis has demonstrated the importance of the C3HC4 zinc RING finger domain of bICP0 and ICP0 (Everett, 1987, 1988; Everett et al., 1993; Inman et al., 2001). ICP0 (Everett et al., 1997, 1999a, b; Maul & Everett, 1994; Maul et al., 1993) and bICP0 (Inman et al., 2001; 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 because ICP0 has E3 ubiquitin ligase activity (Boutell et al., 2002; Van Sant et al., 2001), and it interacts with protein degradation machinery (Everett et al., 1997, 1999a). The E3 ubiquitin ligase activity of ICP0 disrupts the cell cycle and alters cellular gene expression (Hobbs & DeLuca, 1999; Lomonte & Everett, 1999). Apart from the zinc RING finger, bICP0 and ICP0 share little amino acid similarity.

In transient transfection assays, bICP0 is a potent trans-activator of viral promoters, and can regulate certain cellular promoters. For example, bICP0 relieves mad/max mediated transcriptional repression through its association with histone deacetylase 1 (Zhang & Jones, 2001). bICP0 also inhibits the human interferon-β promoter, in part, by sequestering the co-activator p300 (Y. Zhang, G. Henderson & C. Jones, unpublished data). In the absence of other viral
genes, overexpression of bICP0 is toxic (Inman et al., 2001), and in transiently transfected mouse neuroblastoma cells bICP0 activates caspase 3 by an indirect mechanism that ultimately leads to apoptosis (Henderson et al., 2004). Deletion of amino acids spanning 356–676 of bICP0 altered subcellular localization of bICP0 and prevented transactivation of the TK promoter (Inman et al., 2001).

In this study, we generated a panel of bICP0 mutants by random transposon insertion. We identified two domains that were important for transcriptional activation, aa 78–256 and amino acids at or near position 457. Insertion of a transposon into aa 91W reduced the stability of the bICP0 protein in transiently transfected cells. C-terminal amino acids spanning 607–676 contain a nuclear localization signal (NLS). Deletion of the NLS altered the cellular localization of bICP0 and reduced its ability to activate the TK promoter. These results indicate that bICP0 contains at least three functional domains that are important for transactivating viral gene expression, and one domain near the C terminus that promotes nuclear localization.

**METHODS**

**Cells and plasmids.** CV-1 cells (African green monkey kidney cells), mouse neuroblastoma cells (neuro-2A) or human epithelial 293 cells were grown in Earle’s modified Eagle’s medium supplemented with 5 % fetal bovine serum.

The E2.6 plasmid (a gift from M. Schwyzer; Zurich, Switzerland) contains bICP0 coding sequence controlled by the human CMV promoter. Mutagenesis of the bICP0 zinc RING finger was described previously (Inman et al., 2001). The coding regions of the wild-type (wt) bICP0 and the zinc RING finger mutant 13G/51A were inserted into a Flag-tagged expression vector pCMV2C (Stratagene). The resulting plasmids were designated bICP0 or 13G/51A (see Fig. 1b). A C-terminal deletion of bICP0 (ΔICP0) was constructed by deleting the Sal–Xho fragment (aa 356–676) from the Flag-tagged bICP0 construct (see Fig. 1b). Another C-terminal deletion mutant, designated ΔNcoI mutant was generated by deleting the Ncol–Xhol fragment from the Flag-tagged bICP0 construct (see Fig. 4a).

The pMinCAT plasmid contains the TATA box and transcription start site of HSV-TK promoter from nt −32 to +51 upstream of the CAT (chloramphenicol acetyltransferase) gene (Zhang & Jones, 2001).

**Transient expression and Western blot.** 293 cells were transfected with 20 μg bICP0 expression plasmid by calcium phosphate precipitation method (Zhang & Jones, 2001). At 40 h after transfection, cells were collected and suspended in 20 μl lysis buffer (20 mM HEPES pH 7.9, 400 mM KCl, 1 mM MgCl2, 0.2 mM EDTA, 20 % glycerol, 0.5 mM DTT and complete protease inhibitors in one tablet per 10 ml). The lysate was kept on a rotating device for 1 h at 4 °C and centrifuged for 10 min at 4 °C, 15,000 r.p.m. (12,500 g). To the supernatant, 50 μl 5 x sample buffer (250 mM Tris/HCl pH 6-8, 10 % SDS, 25 % mercaptoethanol) was added and the solution boiled for 5 min. The lysate was used for SDS-PAGE. Immunodetection of bICP0 and its mutants was performed with an anti-Flag antibody (Stratagene; catalogue #200471-21).

**Transient transfection and CAT assays.** The pMinCAT reporter construct and bICP0 expression plasmids were co-transfected into CV-1 cells by the calcium phosphate precipitation method. For each transfection, 15 μg reporter plasmid and 1 μg bICP0 expression plasmid were used to form DNA–calcium phosphate co-precipitates. This solution was incubated with CV-1 cells for 12 h and then replaced with fresh medium. After 24 h, cell lysate was prepared by three freeze–thaw cycles in 0-25 M Tris pH 8.0. CAT activity was measured in the presence of 0-2 μCi (7.4 kBq) [3H]chloramphenicol and 0-5 mM acetyl coenzyme A. All forms of chloramphenicol were separated by thin layer chromatography. CAT activity was measured by using a Bio-Rad Molecular Imager FX. The levels of CAT activity were expressed as fold induction relative to the vector control.

**Generation of bICP0 mutant by EZ::TN in-frame linker insertion.** In-frame linker insertion was performed according to the manufacturer’s manual (Epicentre; catalogue #EZ104KN) with some modifications. The BamHI–SaII fragment of bICP0 from pCMV2C-bICP0 was released and cloned into the same restriction sites of pUC19. The resulting plasmid was used as target DNA for transposon insertion. The transposon insertion reaction contains 1 μl EZ::TN 10 × reaction buffer, 250 ng target DNA, 1 μl EZ::TN transposase, 4:5 μl dH2O. The reaction mixture was incubated for 2 h at 37 °C. EZ::TN 10 × stop buffer (1 μl) was added and the reaction mixture was heated for 10 min at 70 °C. Two microlitres of the reaction was used to transform competent E. coli cells (Transfer Max EC100, Epicentre; catalogue #C02810). One-third of a 300 μl transformation reaction was plated on three separate dishes containing different antibiotics (ampicillin, kanamycin or ampicillin/kanamycin). Colonies from the ampicillin/kanamycin plate were randomly picked to extract target DNA for mapping the transposon insertion sites. Mapping was initially performed by digestion with SaII and confirmed by BamHI digestion. Certain clones were then sequenced to determine the precise insertion sites. A panel of BamHI–SaII fragments carrying transposons was religated with the remaining sequences of bICP0 within the Flag-tagged vector.

**Confocal microscopy to examine subcellular localization of bICP0.** In brief, neuro-2A cells in six-well culture dishes were transfected with 2-5 μg of the designated Flag-tagged bICP0 constructs by Superfect (Qiagen; catalogue #301305). After 16 h, cultures were split into eight-well Lab-Tek culture slides. After incubating for 24 h, cultures were fixed in 4 % paraformaldehyde, and then incubated in cold 100 % ethanol at −20 °C for 2 min. After washing three times with PBS, the slides were blocked in 4 % BSA in PBS for 30 min, then incubated with the anti-Flag antibody (1:100) for 2 h at room temperature. The secondary antibody, Cy2 goat anti-mouse IgG (Jackson ImmunResearch Laboratories) (1:100) was added and incubated for 1 h at room temperature in the dark. The images were collected by using a Bio-Rad confocal laser-scanning microscope (MRC-1024ES) with excitation/emission at 488/520 nm.

**Construction of GFP–bICP0 fusions.** The bICP0 expression vector E2.6 was digested with Ncol and then blunted with the Klenow enzyme. The digested plasmid was purified and subjected to a second digestion with SacII. The Ncol–SacII fragment containing amino acids spanning 607–676 was recovered from an agarose gel. A GFP expression vector, phr-GFP-N1 (Stratagene; catalogue #240059), contains a copy of the hrGFP that lacks a translational termination codon inserted upstream of a versatile multiple cloning site, which allows fusion of hrGFP to the N terminus of protein. The Ncol–SacII fragment was cloned into SaII and SacII digested GFP expression vector (the SaII site was first filled-in with Klenow enzyme). The resulting plasmid was designated as GFP–NLS–bICP0. Five micrograms of each GFP vector or GFP–NLS–bICP0 expression plasmid was transfected into neuro-2A cells by Superfect. At 40 h after transfection, cells were fixed in 4 % paraformaldehyde for 10 min and washed with PBS. Subcellular localization of GFP was visualized by using a fluorescence microscope.
RESULTS

Generation of bICP0 transposon mutants

As discussed above, bICP0 has little similarity to ICP0, except for the well-conserved zinc RING finger. Thus, it is difficult to predict where the bICP0 functional domains are located. To identify functional domains within bICP0 coding sequences, we generated a panel of linker insertions by using the EZ: : TN Linker Insertion method. This strategy is based on an in vitro transposon linker insertion method that uses the hyperactive in vitro Tn5 transposase activity to randomly insert the mutagenic linker. The mutagenic linker contains a 1·1 kb kanamycin antibiotic resistance gene flanked by NotI restriction sites. After transposition is performed in vitro, the DNA mixture is used to transform competent E. coli, and kanamycin-resistant clones are identified. Plasmids containing transposons within bICP0 coding sequences were digested with NotI, thus releasing the 1·1 kb kanamycin resistance gene and leaving 57 nt of the transposon. The DNA sequence of the 57 bp encodes 19 aa that are in-frame with all three reading frames on both strands. DNA sequencing was performed to map precisely the site of transposon insertion. Using this strategy, we generated a panel of 15 unique bICP0 mutants (Fig. 1a, for the position of the respective transposon insertions).

DNA fragments containing the respective transposon

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Fig. 1. Generation of bICP0 mutants by EZ: : TN in-frame linker insertion. (a) The bICP0 coding sequences (BamHI–SalI fragment) was cloned into pUC19. An SphI–XhoI fragment of bICP0 coding sequences was also cloned into pBR322. The transposon insertion reaction was performed as described in Methods. The transposon insertion sites were first mapped by restriction endonuclease digestion, and then precise insertion sites identified by DNA sequencing. Fifteen clones with a single transposon insertion were religated with the remaining sequences of bICP0 from the Flag-tagged vectors. The respective mutants were designated A to O, and the numbers in parentheses denote the amino acid number that was disrupted by transposon insertion. The positions of the zinc RING finger, acidic domain, consensus nuclear localization sequence (NLS; KRRR), ATG and bICP0 stop codon are shown. (b) Schematic of the 13G/51A and ΔbICP0 mutant. Two amino acid substitutions were inserted into conserved C’s of the zinc RING finger of bICP0. The ΔbICP0 mutant construct was prepared by digestion of the wt construct with SalI and XhoI, which deleted sequences from aa 357 to 676. The details of these mutants were described previously (Inman et al., 2001). (c) Human 293 cells (2 × 10⁶ cells) were transfected with 15 µg of the transposon bICP0 mutants. At 40 h after transfection, whole-cell lysate was prepared by using high salt extraction buffer as described in Methods. Each lane contained approximately 100 µg protein. Anti-Flag antibody was used to detect the levels of Flag-tagged bICP0 mutant proteins. The β-actin antibody was purchased from Santa Cruz Biotechnology.
insertions were reintroduced into the Flag-tagged CMV expression vector. To test for expression of the respective bICP0 mutant proteins, human 293 cells were transfected with 15 μg of each mutant expression vector, and at 40 h after transfection Western blots were performed using the anti-Flag antibody (Fig. 1c). The 293 cell line was used for this study because we can readily achieve greater than 60% transfection efficiency, and these cells are resistant to the toxic effects of bICP0. Except for mutant B, the rest of the mutants expressed wt levels of the bICP0 protein. Mutant L migrated faster than wt bICP0 when high salt was used to extract bICP0 from transfected cells. As expected, similar levels of β-actin were present in 293 cells after transfection.

Transactivation potential of the bICP0 transposon mutants

To test whether the transposon insertion affected the ability of bICP0 to activate a minimal HSV-1 TK promoter, pMinCAT and each transposon mutant (A to O) were co-transfected into CV-1 cells (Fig. 2). bICP0 can reproducibly activate the TK promoter more than 10-fold (Inman et al., 2001). However, in this study we can readily achieve greater than 60% transfection efficiency, and these cells are resistant to the toxic effects of bICP0. The transposon mutagenesis strategy yielded few mutants expressed wt levels of the bICP0 protein. Mutant L migrated faster than wt bICP0 when high salt was used to extract bICP0 from transfected cells. As expected, similar levels of β-actin were present in 293 cells after transfection.

Subcellular localization of the bICP0 mutants

It is well established that bICP0 localizes to discrete nuclear structures in productively infected and transfected cells (Inman et al., 2001; Parkinson & Everett, 2001). Thus, it was of interest to test whether transposon insertion altered subcellular localization of bICP0. For these studies, confocal microscopy was performed as described previously (Inman et al., 2001). All of the transposon insertion mutants were expressed in the nucleus (Fig. 3). It appeared that some of the mutants (A, D, E, G and K for example) had a diffuse staining pattern within the nucleus relative to wt bICP0. We suggest these mutations block interactions between cellular proteins that are necessary for subnuclear localization. Mutant M, which did not transactivate the TK promoter (Fig. 2), exhibited punctate nuclear staining similar to wt bICP0. These results suggest that the ability of bICP0 to localize to the nucleus was not the only function necessary for transactivating the TK promoter.

Identification of bICP0 sequences necessary for localization to the nucleus

The transposon mutagenesis strategy yielded few mutants in the C-terminal region of bICP0, even when a plasmid that contained just the C terminus of bICP0 was used (SphI–XhoI fragment). Furthermore, all of the transposon mutants expressed a protein that localized to the nucleus (Fig. 3) suggesting that the NLS is near the C terminus of bICP0. Support for this hypothesis comes from previous findings demonstrating that deletion of amino acid sequences contained in the Sall–XhoI fragment resulted in a truncated protein that localized to the cytoplasm (Inman et al., 2001). To localize further bICP0 sequences that promote nuclear localization, an additional deletion mutant was prepared and confocal microscopy performed. Digestion with NcoI and XhoI from the Flag-tagged pCMV2C-bICP0 deleted aa 607–676 (NcoI mutant) (Fig. 4a). As expected, the Flag-tagged protein expressed from the ΔNcoI mutant was slightly smaller than full-length bICP0, but considerably larger than the protein expressed by the ΔbICP0 mutant (Fig. 4b).
Confocal microscopy demonstrated that the protein expressed by the ΔNcoI mutant was primarily expressed in the cytoplasm (Fig. 5a). The ΔC terminus protein (ΔbICP0) was expressed in the cytoplasm of transfected cells whereas wt bICP0 or the 13G/51A protein was expressed in the nucleus, which was consistent with previous studies (Inman et al., 2001). Within sequences deleted from the NcoI mutant, amino acids spanning 622–625 contain a NLS-like core sequence (KRRR) suggesting this region plays a role in nuclear localization.

To confirm there is a functional NLS within the NcoI–XhoI fragment, the sequences encompassing aa 607–676 were fused to the C terminus of the GFP open reading frame.

**Fig. 3.** Localization of bICP0 transposon mutants (A to O) in transfected cells. Neuro-2A cells (5 × 10^5 cells) were transfected with 5 μg of the designated Flag-tagged mutant constructs. After 40 h, confocal microscopy was performed as described previously (Inman et al., 2001). Each panel is representative cells from three independent experiments.

**Fig. 4.** Generation of the NcoI C-terminal deletion mutant. (a) Schematic of wt bICP0 and C-terminal deletion coding sequences. Positions of the zinc RING finger and acidic domain are indicated. The NLS core sequence is from aa 622 to 625. The zinc RING finger mutant (13G/51A) has two well conserved C’s that were mutagenized to G and A, respectively. The ΔbICP0 construct contains a large deletion after aa 356. The 13G/51A and ΔbICP0 constructs were previously described. (b) 293 cells (2 × 10^6 cells) were transfected with 20 μg Flag-tagged bICP0 expression vectors. At 40 h after transfection, whole-cell lysate was prepared and Western blots performed with anti-Flag antibody.
frame (GFP–NLS–bICP0). Fig. 5(b) demonstrated that the GFP-fusion protein expressed by GFP–NLS–bICP0 was present in the nucleus of transfected cells. In contrast, GFP was randomly dispersed in cells following transfection with the blank expression vector, phrGFP-N.

**Transactivation potential of the ΔNcol mutant**

Additional studies were performed to test whether the ΔNcol mutant could activate the minimal TK reporter construct (pMinCAT). CV-1 cells were co-transfected with pMinCAT and the designated bICP0 mutants, and cell lysate was prepared 40 h after transfection. Fig. 6 demonstrated that wt bICP0 activated pMinCAT promoter activity eight- to ninefold, which was expected. The ΔNcol mutant had reduced levels of transcriptional activation relative to wt bICP0. The ΔbICP0 or the 13G/51A mutant had little or no transactivation activity on this promoter, which is consistent with previous studies (Inman et al., 2001). This result demonstrates that nuclear localization was important for maximal activation of the minimal TK promoter.

**DISCUSSION**

In this study, a panel of transposon insertion mutants was constructed to identify bICP0 domains that affect transactivation and nuclear localization. At least two separate domains, in addition to the zinc RING finger, were necessary for transactivation activity. The C terminus of bICP0 contained amino acid sequences that were necessary for nuclear localization, and localization of the bICP0 protein to the nucleus was necessary for maximal transactivation. Our studies suggested that multiple functional domains within bICP0 were necessary for stimulating promoter activity and productive infection.

Transposon insertion within the N-terminal residues of bICP0 (mutants A to H; aa 78–265) had a dramatic effect on transactivation of the TK promoter. Some of the transposon insertions within this domain (A, E, F and G for example) led to altered subnuclear localization, suggesting these mutations altered the secondary structure of the zinc RING finger, or prevented interactions with cellular proteins. The N terminus of HSV-1 ICP0 also contains a promoter-specific transactivation domain (Lium et al., 1998). Mutant B, which is also within this large domain, did not express high levels of protein suggesting these sequences regulate protein stability. Another transactivation domain was identified at or near aa 457 (mutant M). Although mutant M was abundantly expressed in the nucleus of transfected cells, and had punctate staining similar to wt bICP0, it did not transactivate the TK promoter.
Adjacent to the N-terminal transcriptional activation domain is an acidic domain (residues 280–330) that is rich in acidic (Asp and Glu) and hydroxyl (Ser and Thr) amino acids (Fig. 1a) (Wirth et al., 1992). Two transposon insertions (mutant K and L) disrupt the acidic domain, but had no dramatic effect on protein stability or transactivation of the TK promoter. However, the nuclear localization of mutant K or L appeared to be different from wt bICP0, and mutant protein L migrated faster than wt bICP0. Other proteins, GCN4 and GAL4 for example, contain acidic domains that are necessary for stimulating transcription (Mitchell & Tjian, 1989). An analogous acidic domain is not readily apparent in ICP0 coding sequences suggesting this domain has novel functions unique to bICP0. It is also possible that the acidic domain plays a role in activating certain promoters, but not the minimal TK promoter used in this study, or it is only important in certain cell types.

Amino acid sequences located near the C terminus of bICP0 (NcoI–SacII fragment; Fig. 1a) were necessary for localization of bICP0 to the nucleus in transiently transfected cells. Although this plasmid did not transactivate the TK promoter as effectively as wt bICP0, the level of transactivation was higher than the ΔbICP0 construct. We hypothesize that cytoplasmic bICP0 can activate transcription by an unknown mechanism, or that low levels of the protein enter the nucleus. When the NcoI–SacII fragment was fused in-frame with GFP, the GFP signal localized to the nucleus confirming this fragment contained a NLS. An amino acid motif, KRRR, is located between aa 622 and 625, which resembles other basic motifs known to regulate nuclear localization. Classical NLS are categorized as either monopartite, containing a single cluster of basic amino acid residues (K/R), or bipartite, containing two clusters of basic amino acid residues separated by a linker of 10–12 unconserved amino acids (Kalderon et al., 1984; Lanford & Butel, 1984; Richter et al., 1985; Robbins et al., 1991). The SV40 large T antigen NLS (PKKKRKV) is the prototype NLS. Although the KRRR motif does not match the SV40 T NLS, it does resemble a monopartite NLS. There is another NLS-like core motif, RRRRRT (aa 462–467), within bICP0. However, a fragment containing this motif was unable to alter the GFP localization in transfected cells (data not shown). HSV-1 ICP0 also contains a domain that is necessary for nuclear localization, and this region contains a basic NLS core sequence (PRLRR) between residues 501 and 506 (Everett, 1988). ICP0 subcellular localization is influenced by two other HSV IE proteins, ICP4 and ICP27 (Zhu et al., 1994). Future studies will test whether the KRRR motif is important for NLS function, and if viral proteins influence the subcellular localization of bICP0.

In summary, our studies have identified four separate domains that play a role in activating a simple promoter: (i) the zinc RING finger located between aa 13 and 51 (Inman et al., 2001), (ii) a large domain that spans aa 78–265, (iii) sequences at or near aa 457, and (iv) an NLS that is located near the C terminus of bICP0. We have recently developed a bICP0 null mutant virus that grows very poorly in bovine cells (V. Geiser, Y. Zhang & C. Jones, unpublished data). This mutant will be useful to develop additional mutants in the four functional domains that were identified in this study. Since bICP0 is considered to be the major regulatory protein encoded by BoHV-1 (Wirth et al., 1992), understanding the role that bICP0 plays during productive infection and the latency-reactivation cycle is important.

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