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# Identification and Zinc Dependence of the Bovine Herpesvirus 1 Transactivator Protein BICP0

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**Bovine herpesvirus 1 (BHV-1) specifies an unspliced early 2.6-kb RNA (ER2.6) which is 3′ coterminal with exon 2 of the 2.9-kb immediate-early (IE) RNA. The two transcripts have a common open reading frame (676 codons). The predicted protein, designated BHV-1 infected cell protein 0 (BICP0), contains a zinc finger domain with homology to ICP0 of herpes simplex virus type 1 and protein 61 of varicella-zoster virus, and depending on the promoter, it acts as a strong activator or as a repressor in transient expression assays. In situ immunoadsorbent assays using antisera against synthetic oligopeptides demonstrated that BICP0 accumulates in nuclei of BHV-1-infected cells, as expected for an IE gene product involved in gene regulation. Western blots (immunoblots) revealed a BHV-1-specific 97-kDa protein which was detectable during the IE phase and also at later periods of infection, indicating that the kinetics of BICP0 synthesis is consistent with the switch from IER2.9 to ER2.6. To confirm that ER2.6 encoded the 97-kDa BICP0 protein, a DNA fragment containing BICP0-coding sequences was inserted into the *Autographa californica* baculovirus genome. A recombinant protein, identified by its reactivity with antipeptide sera, exhibited the same electrophoretic mobility as BICP0 specified by BHV-1. We microinjected *Xenopus* oocytes with a BICP0 effector plasmid and a promoter-chloramphenicol acetyltransferase plasmid. BICP0-induced stimulation of this promoter was strongly reduced when intracellular zinc was chelated by thionein, indicating that the effect of BICP0 is zinc dependent.**

Of the 20 genes of bovine herpesvirus 1 (BHV-1) which have been mapped and sequenced, four belong to the immediate-early (IE) kinetic class. The IE genes are grouped in two divergent transcription units with start sites located in the inverted repeats (13, 38, 39) (Fig. 1a and b). Transcription unit 2 specifies a 1.7-kb IE RNA (IER1.7) which encodes BHV-1 infected cell protein 22 (BICP22), the homolog of ICP22 of herpes simplex virus type 1 (HSV-1) and related proteins of other herpesviruses (34). Transcription unit 1 specifies three alternatively spliced BHV-1 transcripts, IER4.2, IER2.9, and IER1.5 (*circ*), with a common noncoding leader sequence (exon 1) under the control of a single promoter (13, 38, 39). Whereas IER4.2 is located entirely in the repeat and is therefore diploid, IER2.9 is transcribed over the $IR_S$-$U_L$ junction, and IER1.5 is transcribed over the $TR_S$-$U_L$ junction of the circularized genome. Exon 2 of IER1.5 is 3′ coterminal with a 1.1-kb late RNA (LR1.1), and both encode the 247-amino-acid *circ*-encoded protein which is the homolog of varicella-zoster virus (VZV) protein 2 and equine herpesvirus 1 protein 3 (13). Exon 2 of IER4.2 encodes BICP4, the homolog of HSV-1 ICP4 (33). Exon 2 of IER2.9 is 3′ coterminal with an unspliced 2.6-kb early RNA (ER2.6), and both reveal an identical open reading frame (ORF) with a coding potential of 676 amino acids (38). The predicted protein, designated BICP0 (formerly p135), contains a cysteine-rich zinc finger domain bearing homology to similar domains found in ICP0 of HSV-1 and protein 61 of VZV.

The BICP0, ICP0, and protein 61 genes are located at similar positions on the genome, and all are involved in gene regulation. In transient expression assays, BICP0 acts as a strong activator of a variety of homologous and heterologous promoters, including the IER4.2/2.9 and ER2.6 promoters, and as a repressor of the IER1.7 promoter (38). ICP0 is a general activator in transient expression assays (2, 3, 7, 30) as well as in HSV-1 infections (2, 12). Protein 61, the VZV homolog, directly or indirectly transrepresses the effect of VZV IE transactivating proteins (29), although it has been shown to complement an HSV-1 ICP0 deletion mutant (28). The roles of several ICP0 domains have been determined by mutational analysis (4, 12, 37), and the zinc finger region has been shown to bind zinc (11) and to be important for function (8, 9). The novel type of zinc finger motif was proposed to be named RING finger and was found in 28 viral and cellular regulatory proteins, including the human RING1 gene product (15, 22, 38).

Functional requirement for zinc ions has not yet been shown for any of the RING finger proteins but has been demonstrated in vitro for other classes of zinc finger proteins by using chelating agents (14, 19). As a physiological zinc chelator, the cysteine-rich protein thionein (apometallothionein) was used in an in vitro assay in which it suppressed binding of transcription factor Sp1 to DNA (41). In an in vivo zinc chelation assay, thionein, microinjected into *Xenopus* oocytes, specifically inhibited transcription of 5S RNA via the zinc finger protein TFIIIA (40, 42).

The purpose of this work was to identify BICP0 as a protein. Using antipeptide sera, we identified BICP0 in BHV-1-infected Madin-Darby bovine kidney (MDBK) cells as well as in *Spodoptera frugiperda* (Sf9) cells infected with a recombinant *Autographa californica* baculovirus. Finally, we demonstrated that transactivation by BICP0 depends on zinc ions. The in vivo zinc chelation assay in *Xenopus* oocytes was modified for this purpose by using the CAT (chloramphenicol acetyltransferase)

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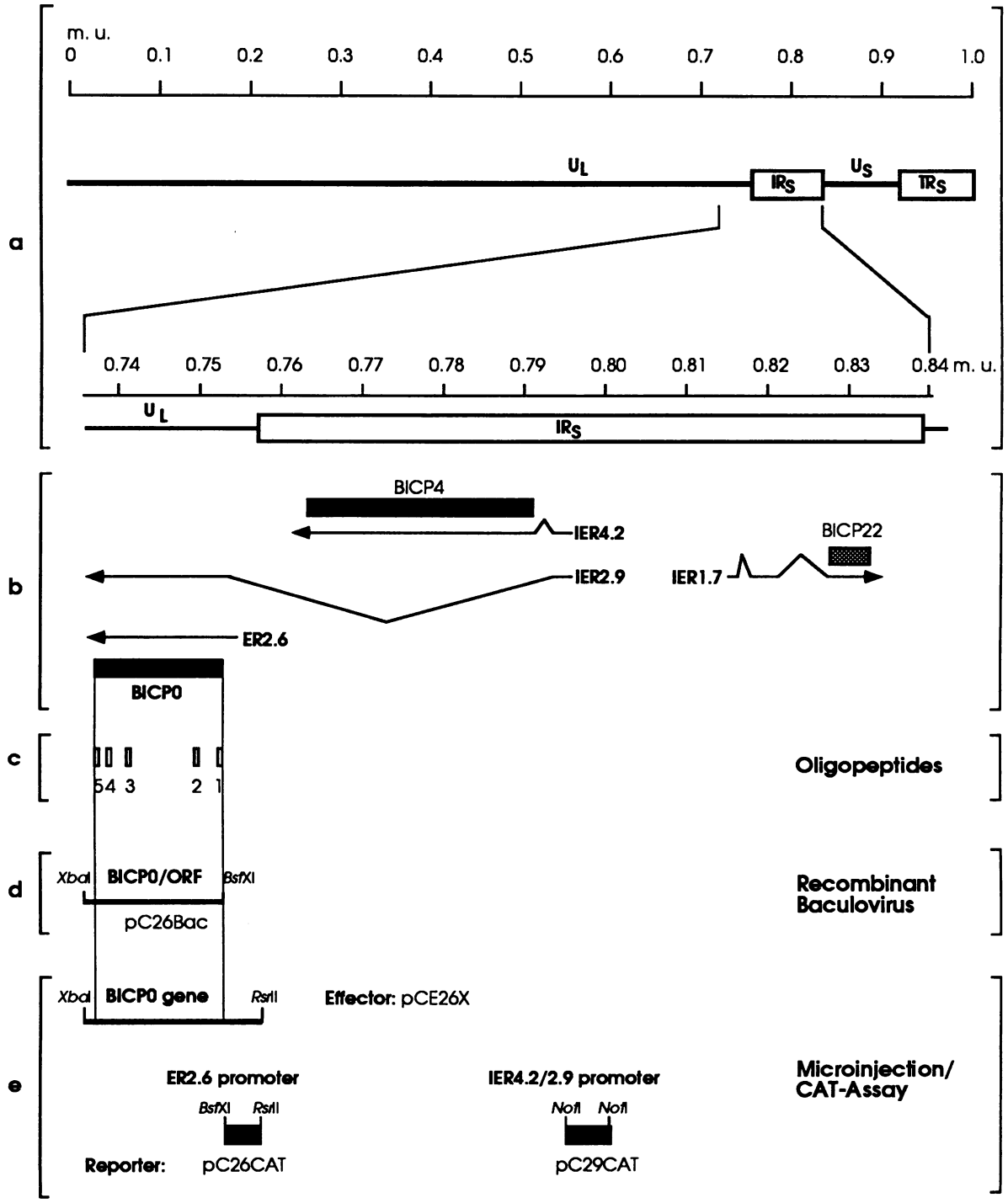


FIG. 1. Summary figure. (a) The standard BHV-1 genome is a linear double-stranded DNA of 138 kbp, subdivided into a unique long segment ($U_L$; 106 kb), a unique short segment ($U_S$; 10 kb), and inverted internal ($IR_S$) and terminal ($TR_S$) repeat sequences (10 kb each) flanking the $U_S$ segment. m.u., map units. (b) Map of BHV-1 transcription and translation products in $IR_S$ and the flanking $U_L$ segment from 0.736 to 0.839 map units. (c) Locations of five oligopeptides (1 to 5) synthesized according to the deduced amino acid sequence of BICP0. The oligopeptides were used for antiserum production in rabbits. (d) Location of the DNA fragment which was inserted into the *A. californica* (PAK-6) baculovirus genome. (e) Locations of DNA fragments used as effector (BICP0 gene) and as reporter (CAT gene under control of the BHV-1 IER4.2/2.9 promoter or the ER2.6 promoter) in a transient expression assay in *Xenopus* oocytes. Restriction enzyme cleavage sites used for subcloning are indicated.

TABLE 1. Synthetic peptides used for antiserum production

| Peptide | Relative location[a] | Sequence[b] | Antiserum |
|---|---|---|---|
| 1 | 1–12 | MAPPAAAPELGS**RRY** | 3, 4 |
| 2 | 179–195 | TPGHGPGAPYLRRVVEW | 5, 6 |
| 3 | 412–425 | **Y**CSPEPREEGRGAGL | 7, 8 |
| 4 | 594–607 | **K**NGNPGRERRPASAM | 9, 10 |
| 5 | 663–676 | GLLGRCSGGSAWRQ | 11, 12 |

[a] Amino acid numbering is based on the first methionine in the BICP0 ORF being designated 1.
[b] Boldface residues were added to facilitate coupling to ovalbumin.

gene as a reporter and may be generally applicable for transeffector proteins which contain a putative zinc finger domain.

## MATERIALS AND METHODS

**Cell cultures and viruses.** MDBK cells were cultured in Eagle's minimal essential medium (Gibco BRL, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS). BHV-1 strain Jura (26) or strain K22 (20), BHV-5 strain N569 (26), and caprine herpesvirus 1 (CapHV-1) strain E/CH (6) were propagated at a multiplicity of infection of 0.01 PFU in MDBK cells in minimal essential medium containing 2% FBS. Sf9 cells (Invitrogen, Heidelberg, Germany) were cultured in complete Grace medium (Serva, Heidelberg, Germany) with 10% FBS. Virus stocks of baculovirus (*A. californica*) strain PAK-6 (Clontech, Palo Alto, Calif.) and recombinant baculoviruses were prepared in Sf9 cells as described by Summers and Smith (36).

**Antipeptide sera.** The deduced amino acid sequence of the BHV-1 BICP0 gene was analyzed with the program Peptidestructure of the University of Wisconsin Genetics Computer Group (5) in order to identify regions likely to be immunogenic. Synthesis of five selected oligopeptides (Fig. 1c and Table 1) and coupling to ovalbumin were performed by a commercial supplier (Neosystem S.A., Strasbourg, France). All cysteines carried an acetamidomethyl protecting group. Peptides (7 mg) were coupled to ovalbumin (6 mg) via the amino terminus by using glutaraldehyde (peptides 2, 4, and 5) or via tyrosine by using bisdiazobenzidine (peptides 1 and 3). New Zealand White rabbits were each subcutaneously injected with 0.75 mg of ovalbumin-coupled peptide in 1 ml of phosphate-buffered saline (PBS) emulsified with an equal volume of Freund's complete adjuvant. Every 14 days, the rabbits received booster injections containing 0.5 mg of ovalbumin-coupled peptide in 1 ml of PBS emulsified with an equal volume of Freund's incomplete adjuvant. Antisera were collected after 10 weeks and screened by in situ immunoadsorbent assays and immunoblotting. Rabbits and sera were designated as shown in Table 1.

**In situ immunoadsorbent (black plaque) assay.** Biotin-avidin-enhanced immunoassays were performed essentially as described by Kousoulas et al. (21) except that cells were fixed with methanol to permeabilize the cell membrane for antibodies. Briefly, monolayers of MDBK cells ($10^5$ cells per well) in a 96-well plate were infected with BHV-1 (Jura or K22), BHV-5, or CapHV-1 at a concentration which resulted in 30 to 50 plaques per well and incubated at 35°C until plaques were visible (usually after 48 h). Then cells were washed twice with PBS containing 5% bovine serum albumin (100 μg per well) and incubated for 30 min at 35°C. After fixation of the cells with methanol, 50 μl of diluted rabbit antipeptide serum per well was added, and plates were incubated again for 30 min at 35°C. Cells were washed three times with PBS and incubated for 30 min at 35°C with 50 μl of biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories Inc., Reactolab S.A., Servion, Switzerland) per well. After washing with PBS, ABC solution (avidin, biotinylated horseradish peroxidase, PBS; Vectastain kit) prepared as instructed by the supplier (Reactolab S.A.) was added to the cells at 50 μl per well. After 30 min at 35°C, the ABC solution was aspirated and cells were washed twice with PBS: then substrate consisting of DAB solution (3,3′-diaminobenzidine tetrahydrochloride; 10 mg/15 ml, of PBS; Sigma) and 0.025% $H_2O_2$ was added at 50 μl per well. When staining was complete, substrate was replaced by PBS.

**Insertion of the BHV-1 BICP0 gene into the PAK-6 genome.** From subclone pCE26X (38), which contains the BHV-1 (strain Jura) BICP0 gene and the polyadenylation signal, a 2.5-kb *Bst*XI fragment was isolated in order to construct a baculovirus transfer plasmid (Fig. 1d). One of the *Bst*XI cleavage sites was located in the multiple cloning site downstream of the polyadenylation signal; the other overlapped the ATG codon in such a way that after removal of 3′ protruding ends with T4 DNA polymerase, the resulting fragment started with nucleotide +1 of the protein-coding sequence. From the resulting plasmid, pBORF26, a 2.5-kb *Bam*HI-*Xba*I fragment was isolated and inserted between the *Bam*HI and *Xba*I sites of the baculovirus transfer plasmid pVL1393 (Invitrogen), forming pC26Bac. The *Bam*HI site is located 42 nucleotides upstream of position +1 of the BICP0-coding sequence, and the *Xba*I site is located 198 nucleotides downstream of the polyadenylation signal. Plasmid DNA and viral DNA isolation and cotransfection into Sf9 cells were performed as described by Summers and Smith (36) except that the viral DNA isolated from baculovirus strain PAK-6 (Clontech) was cut with *Bsu*36I prior to transfection to prevent wild-type DNA from forming infectious progeny. Four days posttransfection, virus progeny was diluted 10 times in complete Grace medium and inoculated onto confluent monolayers of Sf9 cells. After 1 h, an overlay with complete Grace medium containing 1% low-gelling-temperature agarose (Invitrogen) and 10% FBS was added, and plates were incubated at 27°C for 6 days.

**Preparation of cell lysates, polyacrylamide gel electrophoresis, and immunoblotting.** Monolayers of MDBK cells were mock infected or infected with BHV-1 (strain Jura) in either the absence or presence of metabolic inhibitors as described by Misra et al. (27). Sf9 cells were either infected as described by Summers and Smith (36) with PAK-6 or recombinant baculovirus at a multiplicity of infection of 5 PFU or mock infected and incubated at 27°C. Cell extracts for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were prepared as described elsewhere (25). Western blotting (immunoblotting) was performed as described by Friedli and Metzler (16), with some modifications. Proteins were transferred electrophoretically to nitrocellulose sheets and probed with rabbit antisera. The nitrocellulose sheets were incubated with gentle shaking at 37°C with washing buffer (50 mM Tris-HCl [pH 7.4], 140 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40, 0.025% gelatin) containing 10% skim milk. After 5 h, blots were washed twice with washing buffer containing 1% skim milk. Antibody incubation was performed overnight at 4°C in washing buffer supplemented with 1% skim milk and the diluted rabbit antisera. After washing, sheets were incubated for 1 h at 37°C in PBS containing diluted (1:1,000) horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G (Nordic Immunological Laboratories, Tilburgh, The Netherlands). After being washed with PBS, nitrocellulose sheets were transferred to a

substrate solution containing 0.01% 4-chloro-1-naphthol (Sigma) and 0.015% $H_2O_2$ in PBS. When staining was complete, substrate was replaced by $H_2O$.

**DNA injection into *Xenopus* oocytes and CAT assays.** Stage V and VI *Xenopus* oocytes were obtained from anesthetized (MS222; Sandoz) frogs (purchased from H. Kähler, Institut für Entwicklungsbiologie, Bedarf für Forschung und Lehre, Hamburg, Germany). After follicular cells were removed by treatment with collagenase A (2 mg/ml; Boehringer), the oocytes were thoroughly rinsed with modified Barth's solution [MBS; 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)] and maintained at 18°C in MBS containing 0.1 mg of gentamicin per ml. Thionein and zinc-saturated metallothionein ($Zn_7$-thionein) were prepared as described previously (41). All experiments were performed at least three times. Three groups of approximately 300 oocytes each were first injected into the cytoplasm at the vegetal pole with either 80 nl of Tris-HCl (50 mM, pH 7.4), 80 nl of thionein solution (0.5 μg in Tris-HCl), or 80 nl of $Zn_7$-thionein solution (0.5 μg in Tris-HCl). After incubation for 4 h at 18°C in MBS, oocytes (20 to 30 for each DNA) were injected into the nucleus with effector and/or reporter plasmid DNA (50 ng of each in 20 nl of Tris-HCl). Plasmid pCE26X (38) contained the entire BICP0 gene under the control of its native ER2.6 promoter (Fig. 1e) and was used as effector; plasmids pSV2CAT (17), pC29CAT (38), and pC26CAT (38) were used as reporters and contained the CAT gene directed by the simian virus 40 early promoter/enhancer, the BHV-1 IER4.2/2.9 promoter, and the BHV-1 ER2.6 promoter (Fig. 1e), respectively. After nuclear injection, the oocytes were incubated overnight (15 h) at 18°C in MBS and then mechanically lysed in 10 μl of Tris-HCl (250 mM, pH 8), per oocyte. CAT assays were carried out as described previously (38) except that each reaction mixture contained 30 μl of lysate (three oocyte equivalents). Thin-layer chromatograms were evaluated by storage phosphor technology (18). After exposure to the thin-layer plates for 3 h, a phosphor screen was scanned with a PhosphorImager (Molecular Dynamics, Inc.), and the data were processed on a personal computer by using Image Quant and Excel software. Substrate turnover was calculated as radioactivity of the acetylated products divided by the sum of the radioactivities of nonacetylated and acetylated substrate. Alternatively, after injection of DNA, the oocytes were incubated for 15 h at 18°C in MBS containing [$^{35}$S]methionine (15 μCi/ml). Extracts for SDS-polyacrylamide gel electrophoresis were prepared by mechanically lysing the oocytes in SDS-sample buffer (10 μl per oocyte). Total proteins were separated on a 10% polyacrylamide gel (one oocyte equivalent per lane) and electrophoretically transferred to a nitrocellulose sheet. After exposure to the nitrocellulose sheet, a phosphor screen was scanned with a PhosphorImager (Molecular Dynamics), and the intensities of single $^{35}$S-labeled proteins or the overall densities of the lanes were compared.

## RESULTS

As a guide to the experiments described below, Fig. 1 shows the standard BHV-1 genome (Fig. 1a), a map of BHV-1 transcripts and their translation products in $IR_S$ and the flanking $U_L$ segment (Fig. 1b), locations of five oligopeptides which were synthesized according to the deduced amino acid sequence of BICP0 and were used for antiserum production in rabbits (Fig. 1c), the location of the DNA fragment inserted into the *A. californica* (PAK-6) baculovirus genome (Fig. 1d), and locations of DNA fragments used as effector (BICP0 gene) and as reporter (IER4.2/2.9 promoter or ER2.6 promoter linked to a CAT gene) in transient expression assays in *Xenopus* oocytes (Fig. 1e).

**Nuclear accumulation of BICP0 and characterization of antipeptide sera.** In situ immunoadsorbent assays were performed to identify BICP0 and to determine its intracellular distribution. The BICP0 protein was expected to accumulate in cell nuclei, since transient expression assays had shown that this IE protein is involved in gene regulation (38). After 48 h, when plaques became visible, fixed cells were incubated with antipeptide sera as described in Materials and Methods. Sera (dilutions of between 1:600 and 1:1,200) against all five oligopeptides specifically reacted with antigens localized in nuclei of cells infected with BHV-1 strain Jura (Fig. 2) or K22 (not shown), indicating that BICP0 entered the infected cell nucleus like the IE proteins of HSV (32). Sera against oligopeptides 3 and 4 also stained nuclei of cells infected with the more distantly related bovid herpesviruses BHV-5 and CapHV-1 (not shown). Preimmune sera were negative in all assays.

The results summarized in Table 2 indicate that the predicted BICP0 ORF is correct from N to C terminus and that oligopeptides 3 and 4 belong to BICP0 regions which are highly conserved.

**BICP0 is present at IE and later periods of the BHV-1 infection.** Synthesis of IER2.9 and ER2.6, the transcripts encoding BICP0, is controlled by alternative promoters of the IE and early classes, respectively (38). The following experiment was performed to identify BICP0 among the infected cell proteins, to determine its $M_r$, and to test whether the protein was synthesized at IE periods and at later times of the infection. MDBK cells were treated with metabolic inhibitors or untreated and either infected with BHV-1 (Jura) or mock infected as described in Materials and Methods. Cells were harvested 24 h postinoculation (p.i.), and total proteins were separated on an SDS-polyacrylamide gel, electrophoretically blotted onto nitrocellulose sheets, and immunostained with antipeptide serum 11. Positive sera detected BICP0, a 97-kDa IE protein which was synthesized under a cycloheximide-actinomycin D block (Fig. 3a, lane 3) and accumulated to higher amounts (2.5-fold) in the absence of inhibitors (lane 2). The 97-kDa polypeptide was absent from mock-infected cells (lane 1), and preimmune sera did not react with proteins of infected or mock-infected cells (not shown). These results are consistent with IER2.9/ER2.6 transcription kinetics determined in previous studies (38).

**Synthesis of recombinant BICP0 in infected Sf9 cells.** A DNA fragment containing the ORF and the polyadenylation signal common to IER2.9/ER2.6 was inserted into baculovirus as a heterologous vector to confirm that it encoded the 97-kDa BICP0 protein. A baculovirus transfer plasmid was constructed, and recombinant baculoviruses were selected and tested for the ability to produce BICP0 in Sf9 cells as described in Materials and Methods. Cells were harvested 36 h p.i., and total proteins were separated on an SDS-polyacrylamide gel, electrophoretically blotted onto nitrocellulose sheets, and immunostained with diluted antipeptide serum 12. The mutants produced a recombinant BICP0 polypeptide of 97 kDa (Fig. 3b, lane 1). This polypeptide was absent from cells infected with the parental PAK-6 baculovirus (lane 2) or from mock-infected Sf9 cells (lane 3). Preimmune sera did not react with proteins from infected or mock-infected Sf9 cells (not shown). The baculovirus recombinant BICP0 protein had the same apparent $M_r$ as the BHV-1-encoded protein. This result definitely demonstrated that IER2.9/ER2.6 encoded BICP0.

**Zinc-dependent transactivation by BICP0.** Previous studies had demonstrated that BICP0 contains a putative zinc finger

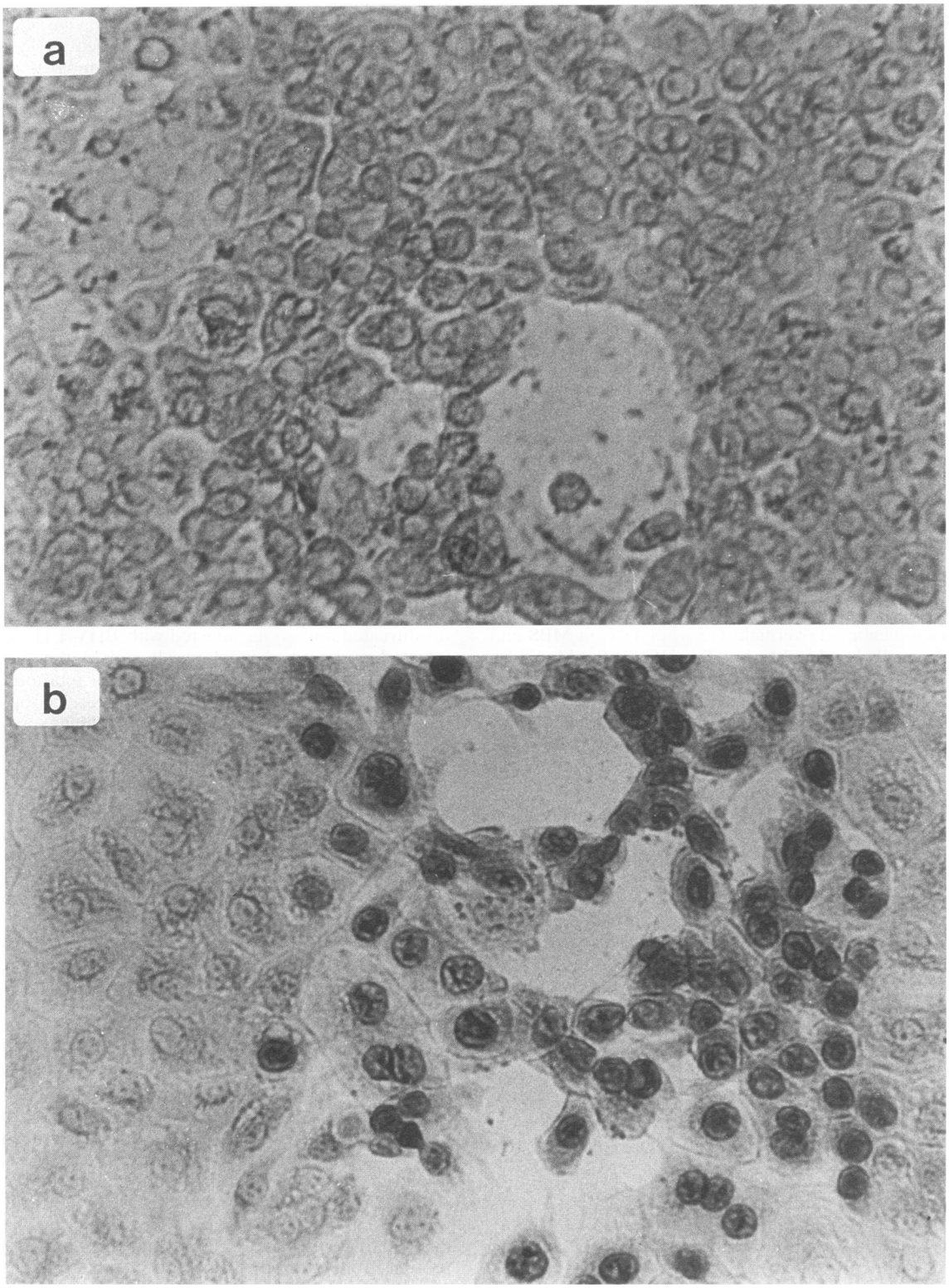


FIG. 2. Photomicrographs showing in situ immunoadsorbent assays of BHV-1 (Jura)-infected MDBK cells. Fixed cells were incubated with preimmune serum 11 (1:400) (a) or with immune serum 11 (1:400) (b) and stained with biotinylated goat anti-rabbit immunoglobulin G.

TABLE 2. Reactivities of rabbit sera prepared against synthetic oligopeptides

| Peptide | Serum | Reactivity | | | | | |
|---|---|---|---|---|---|---|---|
| | | Black plaque assay | | | | Western blotting[a] | |
| | | BHV-1 | | BHV-5 | CapHV-1 | BHV-1 | Recombinant baculovirus |
| | | Jura | K22 | | | | |
| 1 | 3, 4 | + | + | − | − | + | + |
| 2 | 5, 6 | + | + | − | − | + | + |
| 3 | 7, 8 | + | + | + | + | + | + |
| 4 | 9, 10 | + | + | + | + | + | + |
| 5 | 11, 12 | + | + | − | − | + | + |

[a] Of total proteins of BHV-1-infected MDBK cells or Sf9 cells infected with a recombinant baculovirus, as indicated.

domain and acts, depending on the promoter, as a potent activator or as a repressor in transient expression and CAT assays (38). In this study, we attempted to determine whether transactivation by BICP0 depends on zinc ions. Therefore, transient expression assays were performed in oocytes which had been microinjected with thionein to chelate and deplete the intracellular pool of zinc. Oocytes injected with buffer or with zinc-saturated $Zn_7$-thionein served as controls. The three groups of oocytes pretreated in this way were then injected with effector and reporter plasmids, and 15 h later, oocytes were lysed and assayed for CAT activity.

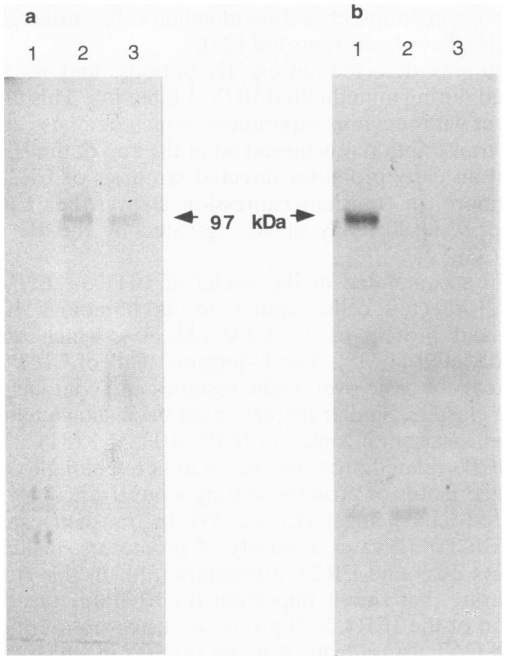


FIG. 3. (a) Immunoblot analysis of BHV-1 (Jura) proteins with immune serum 11 (1:100). MDBK cells were mock infected (lane 1) or infected with BHV-1 either in the absence of metabolic inhibitors (lane 2) or in the presence of 50 μg of cycloheximide per ml (from 1 to 6 h p.i.) and 2.5 μg of actinomycin D per ml (from 7 to 24 h p.i.) (lane 3). Total proteins were harvested 24 h p.i., separated on an SDS–10% polyacrylamide gel ($1.5 \times 10^5$ cell equivalents per slot), and transferred to nitrocellulose. A 97-kDa polypeptide detected by immune serum 11 is indicated (arrow). (b) Immunoblot of recombinant BICP0 with immune serum 12 (1:500). Sf9 cells were infected with either a recombinant baculovirus (lane 1) or parental PAK-6 baculovirus (lane 2) or were mock infected (lane 3). Total proteins were harvested 36 h p.i., separated on an SDS–10% polyacrylamide gel ($2 \times 10^4$ cell equivalents per slot), and blotted onto nitrocellulose. A polypeptide of 97 kDa is indicated (arrow).

The results of these experiments are shown in Fig. 4a and b. BICP0-induced CAT activity was 72-fold greater (Fig. 4a, lane 1; Fig. 4b, bar 1) than the basal CAT activity of pC29CAT (Fig. 4b, bar 5). When $Zn^{2+}$ was removed from oocytes by injection of thionein prior to injection of plasmid DNA, BICP0-induced transactivation was reduced 4.5-fold (Fig. 4a, lane 2; Fig. 4b, bar 2). In contrast, transactivation was only marginally affected (Fig. 4a, lane 3; Fig. 4b, bar 3) when zinc-saturated thionein was injected into the oocytes. To exclude a direct effect of thionein on CAT activity, the extract tested in Fig. 4a, lane 1 (Fig. 4b, bar 1) was also assayed in the presence of thionein (0.5 μg per oocyte equivalent), which did not significantly inhibit the enzyme (Fig. 4b, bar 4).

These results demonstrated that thionein but not zinc-saturated thionein suppressed transactivation of the IER4.2/2.9 promoter by BICP0 and indicated that the presence of zinc ions in the oocytes was necessary for transactivation.

**Zinc depletion by thionein does not cause general effects on protein synthesis in *Xenopus* oocytes.** Previous studies had demonstrated that thionein microinjected into *Xenopus* oocytes specifically inhibited transcription of 5S RNA induced by TFIIIA, a zinc finger protein, but affected neither TFIIIA-independent transcription of a plasmid-encoded $tRNA^{Arg}$ gene by RNA polymerase III nor production of endogenous oocyte mRNA by RNA polymerase II (40, 42). To exclude that thionein might affect protein synthesis in general, we performed a set of control experiments. Oocytes were treated as before except that after the injection of DNA, they were incubated for 15 h in MBS containing [$^{35}$S]methionine (15 μCi/ml). Oocyte lysates were then assayed for (i) CAT activity and (ii) labeled proteins as follows.

(i) CAT assays were performed as in the preceding experiment except that the reaction time was increased from 1 to 2 h in order to obtain better-distinguishable substrate turnovers. Values in the bar graph shown in Fig. 4c are averages of three independent experiments with pCE26X and pC29CAT (bars 1 and 2), pC26CAT (bars 3 and 4), or pSV2CAT (bars 5 and 6) injected into oocytes pretreated with either Tris-HCl (bars 1, 3, and 5) or thionein (bars 2, 4, and 6). As in the previous experiment (Fig. 4a and b), injection of thionein caused a strong reduction in BICP0-induced transactivation relative to the control lacking thionein (bars 1 and 2; ratio of 0.25). In contrast, injection of thionein did not significantly reduce CAT activity generated by either pC26CAT (bars 3 and 4; ratio of 0.83) or pSV2CAT (bars 5 and 6; ratio of 1.15). Since the promoter used in pC26CAT was the same as that used in the effector plasmid pCE26X, the result shown by bars 3 and 4 also excluded the possibility that zinc depletion had an indirect effect on BICP0 synthesis via its own promoter.

(ii) Total proteins of the lysates used for the CAT reactions shown in Fig. 4c were separated on a 10% polyacrylamide gel and blotted onto nitrocellulose. No differences in peak sizes of single $^{35}$S-labeled proteins or in the overall densities of the lanes were observed between lysates of oocytes injected with either thionein or Tris-HCl (data not shown). These results demonstrated that thionein had no general deleterious effects at the level of transcription (ER2.6 or simian virus 40 promoter) or translation (synthesis of endogenous oocyte proteins) and indicated that it had a specific inhibitory effect on BICP0 as a result of zinc depletion.

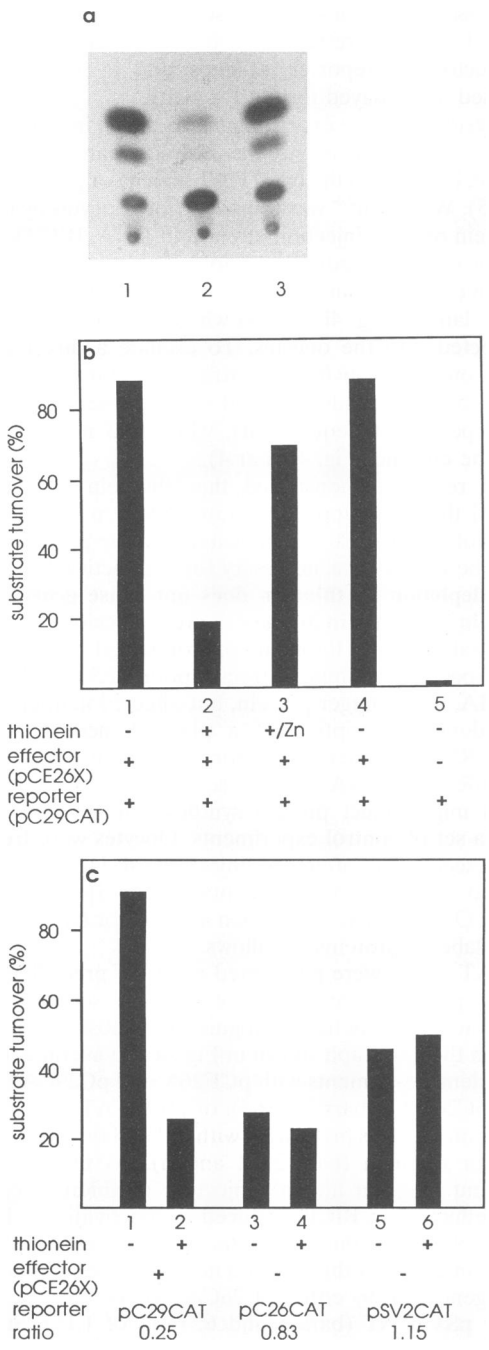


FIG. 4. Zinc-dependent transactivation by BICP0 measured by transient expression assays in microinjected *Xenopus* oocytes. (a) Autoradiograph of a thin-layer chromatogram to visualize CAT activity. Lanes 1 to 3 correspond to bars 1 to 3 in panel b except that they were derived from a single experiment. (b) CAT activities evaluated by exposure of a phosphor screen to the thin-layer chromatograms and expressed as percent substrate turnover (averages of three independent experiments). Oocytes were injected with buffer (bars 1, 4, and 5), 0.5 μg of thionein (bar 2), or 0.5 μg of Zn-saturated thionein (bar 3) prior to coinjection of 50 ng each of pCE26X containing the BICP0 gene and pC29CAT containing the BICP0 IE promoter (bars 1 to 4) or injection of 50 ng of pC29CAT alone (bar 5). To exclude a direct effect of thionein on CAT activity, the extract tested in bar 1 was also assayed in the presence of thionein (0.5 μg per oocyte equivalent; bar 4). (c) Experiments (averages of three) were performed as for panel b except that (i) the oocytes were additionally labeled with [$^{35}$S]methionine (which did not become visible on the chromatograms; see text) and (ii) the CAT assay was run for 2 h. Oocytes were injected with buffer (bars 1, 3, and 5) or 0.5 μg of thionein (bars 2, 4, and 6) prior to coinjection of 50 ng each of pCE26X and pC29CAT (bars 1 and 2) or single injection of pC26CAT containing the BICP0 early promoter (bars 3 and 4) or pSV2CAT containing the simian virus 40 early promoter/enhancer (bars 5 and 6). The ratios of the values of even-numbered to odd-numbered bars are indicated.

## DISCUSSION

IER2.9 and ER2.6 share a common ORF with a coding potential for a protein of 676 amino acids with a calculated molecular weight of 67,701. The predicted protein contains a cysteine-rich zinc finger domain and is designated BICP0 because of structural and functional homologies to ICP0 of HSV-1. In the present work, we raised rabbit sera against synthetic oligopeptides based on the nucleotide sequence of IER2.9/ER2.6 and used them as tools for identification of BICP0 as a protein. We expressed the BICP0 ORF in Sf9 cells infected by a recombinant baculovirus. Finally, we demonstrated that the ability of BICP0 to transactivate the IER4.2/2.9 promoter is dependent on zinc ions.

Metzler (24) designated five BHV-1 IE proteins NSa (180 kDa), NSb (170 kDa), NSc (135 kDa), NSf (98 kDa), and NSg (94 kDa). Misra et al. (27) reported four IE proteins with molecular masses of 180, 166, 93, and 91 kDa, which probably correspond to NSa, NSb, NSf, and NSg, respectively (24). Formerly, IER2.9 and ER2.6 were believed to encode the 135-kDa NSc protein, which was named p135 (38). Our results demonstrated that NSf (98 kDa) rather than NSc represents the product of IER2.9/ER2.6. BICP0 synthesized in Sf9 cells infected with a recombinant baculovirus exhibited no apparent size difference from BICP0 specified by BHV-1. The baculovirus-insect cell expression system provides a eukaryotic environment, and usually similar posttranslational modifications of proteins occur in insect and mammalian cells, although some differences have been reported (23).

BICP0 was detected during IE periods, and its amount increased during noninhibited BHV-1 infection. This finding is consistent with previous experiments which demonstrated that IER2.9 transcription was turned off at the end of the IE period and that an early promoter directed synthesis of ER2.6 (38). Furthermore, in transient expression assays, the IER4.2/2.9 promoter was efficiently down regulated by the IE protein BICP4 (33).

BICP0 accumulated in the nuclei of BHV-1-, BHV-5-, or CapHV-1-infected cells, similar to its homologs ICP0 of HSV-1 and protein 61 of VZV (32, 35), which both are phosphorylated (1, 35). The C-terminal half of BICP0 has a large excess of basic over acidic residues and contains several arginine clusters. Similar features have been shown to serve as a nuclear localization signal in ICP4 of HSV-1 (31).

The ICP0-related proteins discussed here belong to a newly recognized family of proteins sharing a novel zinc finger motif designated RING finger (15, 22, 38). In transient expression assays, BICP0 activates a variety of promoters, including its own IER4.2/2.9 and ER2.6 promoters (38). In this study, we demonstrate that zinc is important for BICP0-induced transactivation of the IER4.2/2.9 promoter, using transient expression and CAT assays from *Xenopus* oocytes in which zinc was chelated by thionein prior to injection of effector and reporter plasmids. Everett et al. (11) have demonstrated that the ICP0 zinc finger domain (codons 105 to 177) and equivalent regions from the homologous proteins of VZV and equine herpesvirus

1, which were expressed in bacteria and purified, stably bind zinc. Hitherto, a functional requirement for $Zn^{2+}$ has not been shown for any members of the RING finger proteins but has been demonstrated in vitro for transcription factor Sp1 (41). Furthermore, it has been shown in *Xenopus* oocytes that transcription of the 5S RNA gene, which is controlled by the classical zinc finger protein TFIIIA, but not transcription of genes which are not controlled by TFIIIA was nearly completely suppressed when thionein was microinjected (40, 42).

So far, it is unknown which BICP0 domains are responsible for transactivation or transrepression. ICP0 and BICP0 exhibit only limited homologies between their predicted amino acid sequences, but they may share some functional domains, including the zinc fingers near the N terminus and acidic domains. The acidic region of ICP0 was initially expected to be the transcriptional activation domain, but deletion of amino acids 223 to 247 did not affect the function of ICP0 (10), whereas the N-terminal part containing the zinc finger motif was shown to be important (4, 8, 9).

Proposed mechanisms for the function of ICP0 homologs remain speculative and might not be uniform among herpesviruses. BICP0 acts, depending on the promoter, as a repressor or as an activator (38). ORF61 down regulates ORF62-induced levels of CAT mRNA (29). On the other hand, sequence-specific DNA binding has not been demonstrated for any of these proteins, and therefore a regulation on the RNA level in contrast to transcriptional regulation cannot be excluded.

The studies described here form the basis for future experiments concerning the function of BICP0 or its homologs. (i) What are the mechanisms for activation or repression? (ii) Do they involve DNA, RNA, or protein binding? (iii) Is zinc required for repression? Purification of recombinant BICP0 for further characterization is in progress. We also plan to create mutated BICP0 forms to investigate the roles of specific domains in activation or repression in transient expression assays as well as in the context of the viral genome.

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