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Identification of the Bovine Herpesvirus 1 *circ* Protein, a Myristylated and Virion-Associated Polypeptide Which Is Not Essential for Virus Replication in Cell Culture

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We have recently reported immediate-early (IE) transcription over covalently joined genome ends of bovine herpesvirus 1 (BHV-1). A spliced 1.5-kb IE RNA (IER1.5) is coterminal with an unspliced 1.1-kb late RNA (LR1.1) which is transcribed from the left end of the genome. Sequence analysis reveals an open reading frame common to IER1.5 and LR1.1 predicted to encode the 247-amino-acid *circ* polypeptide. This paper reports on the identification of *circ* as a protein. Using a rabbit antiserum raised against a synthetic oligopeptide representing the carboxy terminus of the predicted *circ* polypeptide for Western blot (immunoblot) analyses and immunofluorescence assays, we identified a 34-kDa virion-associated protein which accumulated in the cytoplasm of infected cells. To confirm that LR1.1 indeed encoded the 34-kDa polypeptide, we inserted a DNA fragment containing *circ* coding sequences into the *Autographa californica* baculovirus genome. A group of recombinant polypeptides with sizes of 32, 34, and 35 kDa were identified by their reactivity with the antipeptide serum. Chicken egg yolk antibodies raised against total proteins of insect cells infected with the recombinant baculovirus identified the 34-kDa *circ* protein specified by BHV-1. The recombinant *circ* polypeptides and the *circ* protein specified by BHV-1 were both myristylated, as determined by radiolabeling with [^{3}H]myristic acid. It was noted that the *circ* gene could be deleted from the BHV-1 genome without impairing virus replication in cell culture.

Bovine herpesvirus 1 (BHV-1) is a member of the subfamily *Alphaherpesvirinae*. In many respects, it is very similar to the prototype of this subfamily, herpes simplex virus type 1 (HSV-1). The genomes of this virus subfamily can be divided into two covalently linked components, long (L) and short (S), consisting of unique (U) and repeated (R) sequences. In HSV-1, both U_L and U_S components are flanked by inverted repeats. Consequently, both the S and L components can invert relative to each other; thus, DNA extracted from virions consists of four populations, differing in the relative orientations of S and L components (13). In contrast, with BHV-1, only the S component is bracketed by inverted repeats, which are termed internal and terminal repeated sequences (IR_S and TR_S; Fig. 1a). Therefore, the S segment is able to invert, whereas the L component is fixed in its orientation. The biological relevance of inversion of the genome components remains unknown.

Herpesvirus protein synthesis is coordinately regulated and sequentially ordered in a cascade fashion with α or immediate-early (IE), β or early (E), and γ or late (L) phases (14). BHV-1 encodes four IE genes which are grouped in two divergent transcription units starting in the repeats (Fig. 1a). One of the IE transcription units specifies a single spliced transcript, IER1.7, which encodes BICP22 (25); the other specifies three alternatively spliced BHV-1 transcripts, IER4.2, IER2.9, and IER1.5 (*circ*), with a common promoter (7, 28, 29). IER4.2 is located entirely in the repeats and encodes BICP4, the homolog of ICP4 of HSV-1 (24). Exon 2 of IER2.9 is transcribed over the IR_S-U_L junction and is 3′ coterminal with an unspliced 2.6-kb early RNA (ER2.6). Both IER2.9 and ER2.6 encode BICP0, a zinc finger transactivator protein which is the homolog of ICP0 of HSV-1 (8, 28). Most interestingly, exon 2 of IER1.5 is transcribed over the TR_S-U_L junction of the circularized genome and is 3′ coterminal with a 1.1-kb late RNA (LR1.1). IER1.5 and LR1.1 reveal identical open reading frames (ORFs) (7). The predicted 247-amino-acid *circ* protein exhibits homology with varicella-zoster virus (VZV) ORF2 (4) and equine herpesvirus 1 (EHV-1) ORF3 or UL1 (27, 30) but has no homolog in HSV-1.

The amino acid sequence of the predicted *circ* protein contains a myristylation signal. Myristylation is rare among herpesvirus proteins but may be of high biological significance with regard to targeting for cellular compartments, virus assembly, virus infectivity, virus uncoating, receptor binding, signal transduction, and oncogenesis. It was therefore important to identify the *circ* protein and to study its functions in BHV-1.

We identified the *circ* protein 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. We found that the recombinant *circ* polypeptides and the *circ* protein specified by BHV-1 are both modified by myristic acid and that *circ* protein accumulates in the cytoplasm of BHV-1-infected cells. Finally, we substituted a large part of *circ* coding sequences by a β-galactosidase expression cassette and demonstrated that this gene is nonessential for BHV-1 replication in cell culture.

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 (19) was propagated at a multiplicity of infection of 0.01 PFU in MDBK cells in minimal essential medium containing 2% FBS. *S. frugiperda* (Sf9) cells (Invitro-

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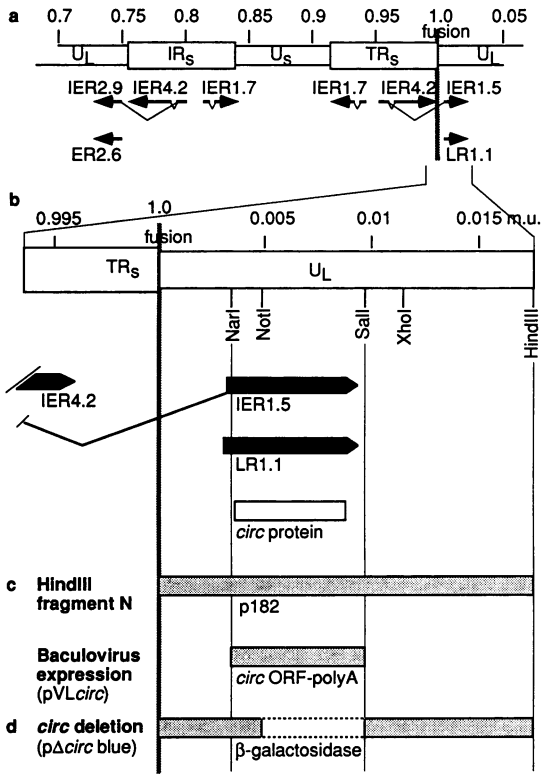

FIG. 1. (a) The S segment of the BHV-1 genome with IR_S, U_S, and TR_S regions, displayed together with adjacent U_L regions. The U_L region attached to TR_S at 1.0 map units (m.u.) represents the left end of the genome, as in a circular or concatemeric molecule. Arrows below represent spliced IE transcripts and unspliced early or late transcripts described in the text. (b) Enlargement of the left end of U_L with relevant restriction sites and maps of transcripts IER1.5 and LR1.1 and the *circ* protein encoded by both transcripts. (c) *Hin*dIII fragment N (2,441 bp) used in constructions shown below. The location of the DNA fragment containing the *circ* ORF and polyadenylation signal (polyA) which was inserted into the *A. californica* (PAK-6) baculovirus genome is shown. (d) Insertion sites of the β-galactosidase expression cassette gene into the *circ* ORF.

gen, 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 (26). A recombinant baculovirus encoding the BHV-1 BICP0 protein (8) was used as a control in this study.

Insertion of the BHV-1 *circ* gene into the PAK-6 genome. Plasmid p182 (5) contains the *Hin*dIII N fragment (0.000 to 0.018 map units) of BHV-1 strain K22 (15) including the entire LR1.1 sequence. A DNA fragment containing the putative *circ* ORF and polyadenylation signal (Fig. 1c) was inserted into the genome of PAK-6 baculovirus as follows. First, p182 was partially digested with *Xho*I and *Nar*I, and the 1,130-bp fragment was isolated and cloned between the *Cla*I and *Xho*I sites of pBsKS+. From the resulting plasmid, pBORF*circ*, a 850-bp *Sal*I-*Eco*RI fragment was excised, blunt end repaired, and inserted into the *Sma*I site of pVL1393 (Invitrogen), forming transfer plasmid pVL*circ*. This plasmid contained the BHV-1 *circ* ORF and polyadenylation signal downstream of the baculovirus polyhedrin promoter. Cotransfection of baculovirus strain PAK-6 genomic DNA with pVL*circ* and isolation of recombinants were performed as recently described (8). One isolate, designated PAK-*circ*, was selected for further analyses.

Immunological reagents. The *circ* antiserum was prepared as follows. Synthesis of an oligopeptide representing the C terminus of the predicted *circ* amino acid sequence (amino acids 232 to 247; SPGALSRYSSVRSVFF) and coupling to ovalbumin were performed by a commercial supplier (Neosystem S. A., Strasbourg, France). Two 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. Antiserum was collected after 6 weeks and designated *circ* antiserum.

BICP0 antiserum has been described previously (8); BICP22 antiserum was obtained from René Köppel. For detection of BHV-1 gB, monoclonal antibody 60 (9) was used. TrpE-UL1 antiserum (12) directed against the UL1 protein of EHV-1 was kindly provided by Ronald N. Harty. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) and horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-chicken IgG, and rabbit anti-mouse IgG were purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands).

Production of chicken egg yolk antibodies. Sf9 cells were infected with PAK-*circ* at a multiplicity of infection of 5 PFU and incubated at 27°C. After 48 h, cells were washed twice with PBS, lysed in a buffer consisting of 25 mM CAPS [3-(chloramidopropyl-dimethylamino)-2-hydroxy-1-propanesulfonate] and 50 mM NaCl (pH 9.5), and the quantity of total protein in the lysate was determined by the method of Bradford (3). Two laying hens (purchased from a local farmer) were each injected at two sites into the pectoral muscle with 30 μg of total PAK-*circ* infected cell protein in 0.75 ml of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.1 M NaCl emulsified with an equal volume of complete Freund's adjuvant. After 14 and 24 days, the hens received booster injections with identical emulsions as described above. Eggs were collected daily and stored at 4°C until use. Extraction and purification of antibodies were performed as described previously (10).

Infection of cells for indirect immunofluorescence assays. Monolayers of MDBK cells (10^5 cells per well) in Lab-Tek tissue culture chamber slides (Miles Scientific, Naperville, Ill.) were infected with BHV-1 at a concentration of 30 to 50 plaques per well. After 12 h, when plaques became visible, cells were washed twice with PBS. After fixation of the cells with methanol for 30 min at room temperature, 100 μl of diluted antiserum (1:100 in PBS) per well was added, and the chamber slides were incubated for 1 h at 37°C in a humid atmosphere. Cells were rinsed extensively with PBS and incubated for 1 h at 37°C with 100 μl of diluted FITC-conjugated goat anti-rabbit IgG (1:100 in PBS). After being washed with PBS, the slides were examined for fluorescence with a Leitz Diaplan fluorescence microscope (Leitz, Wetzlar, Germany) equipped with a 25×/0.75 PL-Fluotar objective and a filter for light with a wavelength of 450 to 490 nm. Photographs were taken with Kodak Ektachrome 1600 film.

Preparation of cell lysates and purification of BHV-1 virions for Western (immunoblot) analyses. MDBK cells or Sf9 cells were either mock infected or infected with BHV-1 or baculovirus, respectively, as described previously (8), except that for some experiments, 20 μCi of [^{3}H]myristic acid (Amersham)

per ml of medium was added from 0 to 24 h postinfection (p.i.) with BHV-1 or from 0 to 36 h p.i. with baculovirus. Purified BHV-1 virions were kindly provided by Vikram Misra. Virions of rBHVΔ*circ*blue (see below) were purified as described by Misra et al. (21). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (8); antibody dilutions used in each immunoblot are indicated in the appropriate figure legends.

Deletion of *circ* from the BHV-1 genome. (i) Plasmid construction. In plasmid p182, a *Not*I cleavage site is located 155 nucleotides after the start codon and a *Sal*I site is located 106 nucleotides after the stop codon of the *circ* ORF (Fig. 1d). After digestion of p182 with *Not*I and *Sal*I, the 4.3-kb fragment was isolated, blunt end repaired with T4 DNA polymerase, and then used as a vector for the insertion of a β-galactosidase expression cassette. The insert was prepared from plasmid pEC10 (kindly provided by Leonard J. Bello), which contains the β-galactosidase gene under the control of the BHV-1 gB promoter. Plasmid pEC10 was digested with *Hin*dIII, and the 3.8-kb fragment containing the β-galactosidase gene was isolated, blunt end repaired with T4 DNA polymerase, and ligated with the vector prepared as described above. The resulting plasmid, pΔ*circ*blue, was used as the transfer plasmid for homologous recombination with BHV-1 genomic DNA to substitute a large part of the *circ* ORF by the β-galactosidase gene.

(ii) DNA transfection. The procedure for preparation of infectious BHV-1 DNA has been described in detail elsewhere (1a, 16, 20). MDBK cells (10^6 per flask) were seeded in 25-cm^2 culture flasks and cotransfected after 24 h with infectious BHV-1 DNA and plasmid DNA, using the calcium phosphate method of Graham and van der Eb (11) as modified by Bello and Lawrence (1). Briefly, 8 μg of viral DNA and 16 μg of pΔ*circ*blue were diluted in HeBS-buffer (0.14 M NaCl, 0.75 mM Na_2HPO_4, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.1]) to a final volume of 379 μl. After addition of 21 μl of 2.5 M $CaCl_2$, the suspension was incubated at room temperature for 30 min. Then, the medium was removed and replaced by 400 μl of the DNA precipitate. After incubation for 30 min at 37°C, 4 ml of Dulbecco's modified Eagle medium (DMEM) was added, and the cells were incubated for 6 h at 37°C. After treatment for 4 min with 30% dimethyl sulfoxide (in HeBS buffer), cells were washed with DMEM and incubated at 37°C until the cytopathic effect was 100% (4 to 5 days posttransfection).

(iii) Screening for *lacZ*$^+$/*circ*-deletion recombinants. Virus progeny from the cotransfection was harvested, plated on confluent MDBK cells, overlaid with DMEM containing 0.75% agarose (Invitrogen) and 300 μg of Bluo-gal (Gibco) per ml, and incubated at 37°C. Blue plaques (usually appearing after 3 to 5 days) were picked and plaque purified three times. One of these substitution mutants, designated rBHVΔ*circ*blue, was selected for further characterization.

RESULTS

As a guide to the experiments described below, Fig. 1 shows part of the BHV-1 genome with covalently joined termini and the transcription pattern of the IE gene region (Fig. 1a), a map of BHV-1 transcripts and their translation product encoded by the left genome end (Fig. 1b), the location of the DNA fragment inserted into the *A. californica* (PAK-6) baculovirus genome (Fig. 1c), and the insertion sites of a β-galactosidase gene substituted for the *circ* ORF (Fig. 1d).

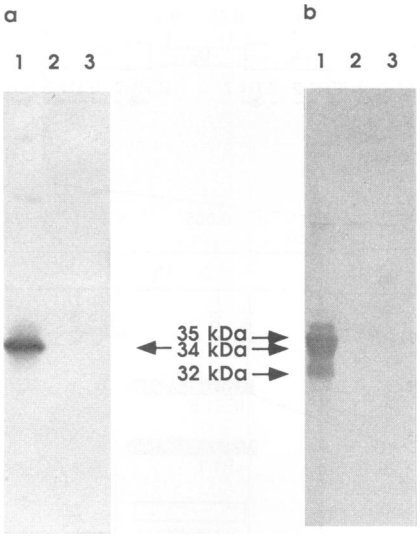

FIG. 2. (a) Immunoblot analyses of BHV-1 proteins with the *circ* antiserum. MDBK cells were infected with either BHV-1 (lane 1) or rBHVΔ*circ*blue (lane 3) or were mock infected (lane 2); total proteins were harvested 24 h p.i., separated on an SDS–10% polyacrylamide gel (1.5×10^5 cell equivalents per slot), and transferred to nitrocellulose. A 34-kDa polypeptide detected by the *circ* antiserum (1:500) is indicated (arrow). (b) Immunoblot of recombinant *circ* protein with the *circ* antiserum (1:500). Sf9 cells were infected with either PAK-*circ* (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 (2×10^4 cell equivalents per slot), and blotted onto nitrocellulose. Recombinant polypeptides with sizes of 32, 34, and 35 kDa are indicated (arrows).

The *circ* antiserum reacts with a 34-kDa BHV-1-specific protein which accumulates in the cytoplasm of infected cells. Western blot analyses and immunofluorescence assays with a rabbit serum raised against a synthetic oligopeptide representing the C terminus of the *circ* polypeptide were performed to identify the *circ* protein among the BHV-1-infected cell proteins and to determine its molecular weight and intracellular distribution. MDBK cells were mock infected or infected with BHV-1. Immunoblot analyses of these cell extracts revealed a 34-kDa BHV-1-specific protein that reacted with the *circ* antiserum (Fig. 2a, lane 1). The 34-kDa protein was absent from mock-infected cells (lane 2), and preimmune serum did not react with proteins of either infected or mock-infected cells (not shown). For immunofluorescence assays, MDBK cells were infected, after 12 h fixed with methanol, and incubated with antiserum as described in Materials and Methods. The *circ* antiserum specifically reacted with antigens localized in the cytoplasm of cells infected with BHV-1 (Fig. 3a). Infected cells exposed to preimmune serum (Fig. 3b) or mock-infected cells exposed to the *circ* antiserum (not shown) did not react in fluorescence assays above background levels.

Synthesis of recombinant *circ* protein in infected Sf9 cells. A DNA fragment containing the ORF and polyadenylation signal common to IER1.5 and LR1.1 (Fig. 1c) was inserted into baculovirus as a heterologous vector to confirm that it encoded the 34-kDa *circ* protein. A baculovirus transfer plasmid was constructed, and 10 recombinant baculoviruses were isolated and tested for the ability to produce the *circ* protein in Sf9 cells. One of the isolates was selected for further characterization and was designated PAK-*circ*. Immunoblot analyses revealed that PAK-*circ* produced a group of polypeptides with

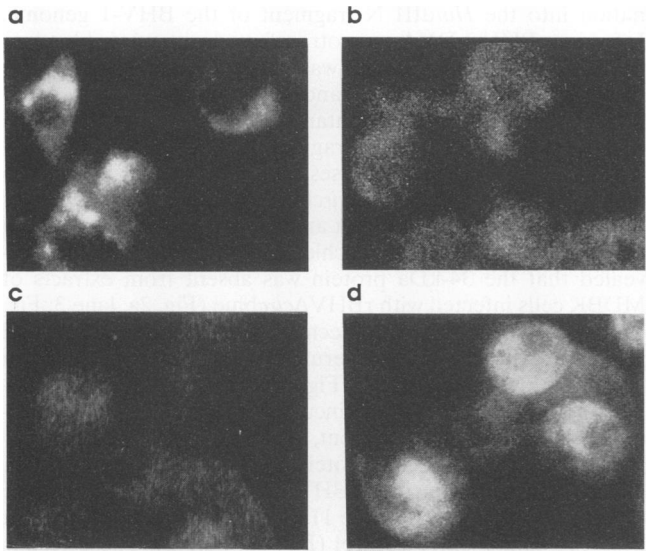

FIG. 3. Photomicrographs showing immunofluorescence assays of BHV-1-infected MDBK cells. Cells were infected with either BHV-1 (a and b) or rBHVΔ*circ*blue (c and d). After 12 h, fixed cells were incubated with the *circ* antiserum (a and c), with preimmune serum (b), or with the BICP0 antiserum (d) and stained with FITC-conjugated goat anti-rabbit IgG. All antibodies used in this experiment were diluted 1:100 in PBS.

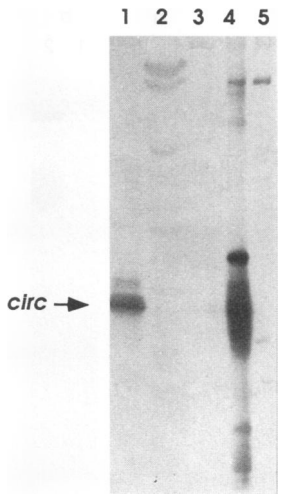

FIG. 4. Immunoblot of BHV-1 and baculovirus proteins with chicken egg yolk antibodies raised against a crude lysate of PAK-*circ*-infected cells. MDBK cells were infected with either BHV-1 (lane 1) or rBHVΔ*circ*blue (lane 3) or were mock infected (lane 2), and Sf9 cells were either infected with PAK-*circ* (lane 4) or mock infected (lane 5). Total proteins were harvested, separated on an SDS–10% polyacrylamide gel, blotted onto a nitrocellulose sheet, and incubated with chicken egg yolk antibodies (1:200). The 34-kDa *circ* protein is indicated (arrow).

sizes of 32, 34, and 35 kDa (Fig. 2b, lane 1) which were absent from cells infected with the parental PAK-6 baculovirus (Fig. 2b, lane 2) or from mock-infected Sf9 cells (lane 3). It should be noted that in order to display well-resolved bands in Fig. 2b, fewer cell equivalents were loaded on the gel compared with Fig. 2a, and the blot was stained less intensely. Large amounts of *circ*-specific polypeptides had been previously shown to accumulate in PAK-*circ*-infected Sf9 cells between 24 and 48 h p.i., reaching up to 20% of total cellular protein, a level at least 100-fold higher than in BHV-1-infected cells (6).

Chicken egg yolk antibodies raised against baculovirus encoded *circ* protein. The following experiment aimed to ascertain the relationship between the baculovirus-encoded *circ* polypeptides and the *circ* protein specified by BHV-1. Sf9 cells were infected with PAK-*circ*, cell extracts were prepared for immunization of hens, and egg yolk antibodies were purified as outlined in Materials and Methods. These antibodies identified the 34-kDa *circ* protein specified by BHV-1 (Fig. 4, lane 1) but did not react with extracts from mock-infected MDBK cells (lane 2). In extracts from PAK-*circ*-infected Sf9 cells (lane 4), they detected the same group of polypeptides as did the rabbit antipeptide antibodies (Fig. 2b, lane 1). Additional bands probably represent unrelated proteins in the crude extracts used to immunize the hens.

The *circ* polypeptides specified by BHV-1 and by a recombinant baculovirus are modified by myristic acid. Computer analyses of the *circ* amino acid sequence predicted a consensus myristylation signal at the N terminus of the protein. This signal is also conserved in EHV-1 ORF3 (UL1), for which myristylation has been already demonstrated (12). The following experiment was performed to demonstrate that the *circ* protein is modified by myristic acid like its EHV-1 homolog. MDBK cells were mock infected or infected with BHV-1 in the presence of [^{3}H]myristic acid. Sf9 cells were mock infected or infected with recombinant baculovirus encoding either the *circ* protein or BICP0 in the presence of [^{3}H]myristic acid. Total proteins were separated on an SDS-polyacrylamide gel and electrically transferred onto a nitrocellulose sheet. Among several radiolabeled proteins detected by autoradiography, a 34-kDa protein was clearly present in extracts of cells infected with BHV-1 (Fig. 5a, lane 1) but was absent from mock-

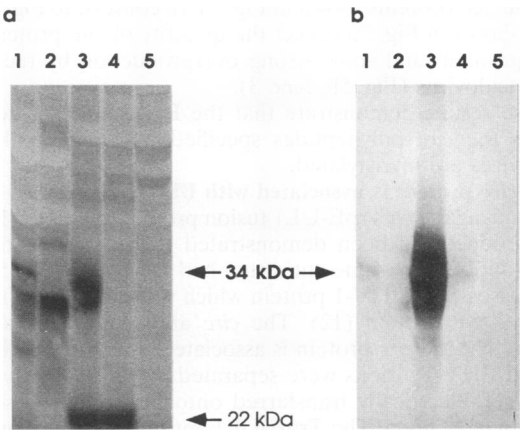

FIG. 5. (a) Autoradiograph showing myristylated proteins of BHV-1-infected MDBK cells and PAK-*circ*-infected Sf9 cells. MDBK cells were either infected with BHV-1 (lane 1) or mock infected (lane 2) in the presence of 20 μCi of [^{3}H]myristic acid per ml (0 to 24 h p.i.), and Sf9 cells were infected with either PAK-*circ* (lane 3) or a recombinant baculovirus encoding BICP0 (lane 4) or were mock infected (lane 5) in the presence of 20 μCi of [^{3}H]myristic acid per ml (0 to 24 h p.i.). Total proteins were resolved on an SDS–10% polyacrylamide gel and transferred to nitrocellulose. Labeled proteins were revealed by exposure of (^{3}H)Hyperfilm (Amersham). (b) Thereafter, the blot was immunostained with *circ* antiserum (1:500). Positions of the *circ* bands (34 kDa) and a band representing a 22-kDa myristylated baculovirus protein are indicated (arrows).

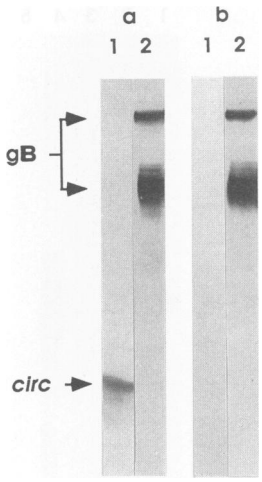

FIG. 6. Immunoblot of BHV-1 virions. Purified BHV-1 virions (a) or rBHVΔ*circ*blue virions (b) were fractionated on an SDS–10% polyacrylamide gel and transferred to nitrocellulose, and identical strips were immunostained with the *circ* antiserum (lane 1) or monoclonal antibody 60 (lane 2). The positions of bands representing the *circ* protein and glycoprotein B are indicated (arrows).

infected cells (lane 2). A diffuse but strong signal was also visible in cells infected with PAK-*circ* (lane 3). This signal was absent from cells infected with the recombinant baculovirus encoding BICP0 (lane 4) used as a control or from mock-infected Sf9 cells (lane 5). The strong signal in lanes 3 and 4 represents a 22-kDa myristylated baculovirus protein (2). As an additional control, the nitrocellulose sheet was also reacted with the *circ* antiserum, which identified the BHV-1 *circ* protein (Fig. 5b, lane 1) as well as the recombinant *circ* polypeptides (lane 3) at sizes which correspond to those of the radiolabeled proteins shown in Fig. 5a. In contrast to Fig. 2, the bands shown in Fig. 5b reflect the quantity of *circ* protein per cell equivalent and show strong overproduction by recombinant baculovirus (Fig. 5b, lane 3).

These results demonstrate that the BHV-1 *circ* protein as well as the *circ* polypeptides specified by the recombinant baculovirus are myristylated.

The *circ* protein is associated with BHV-1 virions. By using antiserum against a TrpE-UL1 fusion protein, the EHV-1 UL1 gene product had been demonstrated to be associated with EHV-1 virions; the same antiserum had reacted with a 35-kDa virion associated BHV-1 protein which was believed to represent the *circ* protein (12). The *circ* antiserum was used to confirm that the *circ* protein is associated with BHV-1 virions. Purified BHV-1 virions were separated on an SDS-polyacrylamide gel, electrically transferred onto nitrocellulose sheets, and immunostained. The TrpE-UL1 antiserum (not shown) as well as the *circ* antiserum clearly identified the 34-kDa *circ* protein as a virion component (Fig. 6a, lane 1). An identical strip was incubated with monoclonal antibody 60, which detected BHV-1 gB (Fig. 6a, lane 2). On the other hand, the BICP0 antiserum did not react with any protein (not shown). These results confirmed that the *circ* protein is associated with BHV-1 virions, whereas BICP0 is not.

A BHV-1 recombinant containing a deletion within the *circ* gene is not impaired for replication in cell culture. Plasmid pΔ*circ*blue contained a β-galactosidase gene in place of a large part of *circ* coding sequences (Fig. 1d) and was flanked by BHV-1 sequences designed to facilitate homologous recombination into the *Hin*dIII N fragment of the BHV-1 genome. Infectious BHV-1 DNA was cotransfected with pΔ*circ*blue into MDBK cells. Virus progeny was harvested and screened for recombinants staining blue, and four selected plaques were purified. The substitution mutants were first checked for the absence of a specific DNA fragment within the *circ* ORF by Southern blot and PCR analyses (data not shown). One of the mutants, designated rBHVΔ*circ*blue, was selected for further characterization. Western blot analyses with the *circ* antiserum or the *circ* protein-specific chicken egg yolk antibodies revealed that the 34-kDa protein was absent from extracts of MDBK cells infected with rBHVΔ*circ*blue (Fig. 2a, lane 3; Fig. 4, lane 3). MDBK cells infected with rBHVΔ*circ*blue and incubated with the *circ* antiserum did not yield any signal in immunofluorescence assays (Fig. 3c). When rBHVΔ*circ*blue-infected MDBK cells were incubated with the BICP0 antiserum in a control experiment, a strong fluorescence representing the BHV-1 BICP0 protein appeared in the nuclei (Fig. 3d). Furthermore, purified rBHVΔ*circ*blue virions lacked the *circ* polypeptide (Fig. 6b, lane 1). In this instance, glycoprotein B served as a positive control (Fig. 6b, lane 2). These results again verified that the 34-kDa protein is encoded by the *circ* gene. Since the deletion mutants yielded normal titers, *circ* appears to be nonessential for virus replication in cell culture.

DISCUSSION

Recent molecular analysis of the BHV-1 genome (23) indicates that it has many similarities to that of the prototype virus HSV-1 with respect to gene organization and homologies of encoded proteins. Nevertheless, BHV-1 exhibits several interesting features which are so far unique within the subfamily *Alphaherpesvirinae*. One of the two BHV-1 1E promoters directs synthesis of three alternatively spliced transcripts (7, 28). Notably, one of those RNAs, IER1.5, arises from transcription over the circularized genome. The predicted amino acid sequence of the putative *circ* protein exhibits strong homology to VZV ORF2 and EHV-1 ORF3 (UL1) but has no homolog in HSV-1 (7). An alternative promoter directs synthesis of a late unspliced RNA, LR1.1, which is 3′ coterminal with IER1.5 and which thus allows synthesis of *circ* protein at late times of infection.

The salient features of the results presented in this study are as follows. (i) The *circ* protein is a 34-kDa polypeptide which accumulates in the cytoplasm of BHV-1-infected cells. It is myristylated and associated with BHV-1 virions. Recently Harty et al. (12) reported on the identification of the UL1 protein of EHV-1 by using polyclonal rabbit antibodies raised against a TrpE-UL1 fusion protein synthesized in *Escherichia coli*. The UL1 protein was a 33-kDa polypeptide with properties similar to those of the *circ* protein described in this report. Although only a few stretches of consecutive identical amino acids can be detected upon alignment of the predicted amino acid sequences for the UL1 product and *circ*, the UL1-specific rabbit serum cross-reacted with a 35-kDa virion-associated BHV-1 protein which was suggested to represent the *circ* protein (12). Our observations confirm this hypothesis.

(ii) Expression of the *circ* gene from a recombinant baculovirus resulted in three recombinant proteins of 32, 34, and 35 kDa, all of which appeared to be modified by myristic acid. The reasons for these different forms of the *circ* protein remain unknown, and the possibility of other modifications, e.g., phosphorylation (the *circ* amino acid sequence reveals many putative phosphorylation sites), remains to be analyzed.

(iii) The functional properties of *circ* and its homologs are unknown. Nevertheless, the present knowledge of the map

location of the gene and the structural properties of the *circ* protein and UL1 allow speculation about their functions. The presence of genes with homology to *circ* in the class D genomes (22) of BHV-1, VZV, and EHV-1, as opposed to absence of *circ* homologs from class E genomes (e.g., HSV-1), indicates that its function may be connected with the genome structure. Since VZV and EHV-1, like BHV-1, have a fixed L segment, one could postulate that gene expression over joined genome ends may functionally complement for genome inversion in these viruses. However, transcription over the TR_S-U_L junction of the genomes of VZV and EHV-1 has not yet been demonstrated. As another possibility, UL1 and *circ* could play a role in virus assembly (12). The UL1 protein was more abundant in cells infected with defective interfering particle-enriched virus than in cells infected with standard EHV-1 (12) and thus seemed to have an important function in those particles. The construction of a recombinant BHV-1, in which 80% of the *circ* coding sequences had been replaced by the β-galactosidase gene, argues against such a hypothesis. However, the fact that *circ* is expressed at high levels may argue for an important in vivo function. Myristylation is a rare modification of herpesvirus proteins (17, 18). Physically, this type of processing allows close association of proteins and membranes. Targeting for specific cellular compartments and participation in virus assembly, virus infectivity, and virus uncoating are well known functions of myristylated viral proteins (18). Furthermore, they have been shown to play roles in receptor binding, signal transduction, and oncogenesis. In vivo experimentation is required to test which explanation may be true.

The constructs and reagents described in this report will serve as useful tools for isolation and biochemical characterization of the *circ* protein. We are now planning to compare the properties of the *circ*-deletion mutant (rBHVΔ*circ*blue) with wild-type BHV-1 in vivo in order to shed more light on the potential role of the *circ* protein in the pathogenesis of BHV-1 infections.

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