Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

ELSEVIER

Virus Research 61 (1999) 29–41

Virus Research

Growth behavior of bovine herpesvirus-1 in permissive and semi-permissive cells

Takayuki Murata [a,c], Yasuhiro Takashima [a], Xuen Xuan [b], Haruki Otsuka [a,*]

[a] *Department of Global Agricultural Sciences, Graduate School of Agricultural and Life Science, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan*

[b] *The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan*

[c] *Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Showa-ku, Nagoya 466-8550, Japan*

Received 22 July 1998; received in revised form 2 February 1999; accepted 5 March 1999

Abstract

Bovine herpesvirus-1 (BHV-1) can replicate well in bovine-derived cell lines such as Madin Darby bovine kidney (MDBK) but grows poorly in hamster lung (HmLu-1). Virus replication, DNA synthesis, and immediate-early gene expression are severely restricted in HmLu-1. We compared adsorption and penetration of BHV-1 in permissive MDBK and semi-permissive HmLu-1 cells. At a low multiplicity of infection, BHV-1 attached to permissive MDBK cells twice as much as to HmLu-1. The presence of heparin inhibited the attachment of BHV-1 to MDBK cells by about 60%, but over 90% of the attachment was inhibited in HmLu-1. To investigate the penetration of BHV-1, we performed the quantitative measurement of viral DNA by quantitative competitive (QC)PCR in infected cells. In MDBK cells, virions attached to the cell surface, penetrated into the cells and were transported to the nucleus. However in HmLu-1, only a small fraction of the virions attached to the cell surface were allowed to penetrate. Our results indicated that the replication of BHV-1 in semi-permissive HmLu-1 was not dramatically restricted at one certain point but at some various stages including adsorption and penetration.

Keywords: Bovine herpesvirus-1; Immediate early protein; Adsorption; Penetration; Quantitative competitive PCR

1. Introduction

Some α-herpesviruses such as herpes simplex virus (HSV) and pseudorabies virus (PRV) have a broad host cell range in vitro. Many cell lines derived from a wide variety of animals, such as human, monkeys and rodents, are permissive to HSV and PRV, and most of the research on these

* Corresponding author. Tel.: +81-3-5841-5191, ext. 7052; fax; +81-3-5841-5191.

E-mail address: aotsuka@hongo.ecc.u-tokyo.ac.jp (H. Otsuka)

0168-1702/99/$ - see front matter
PII: S0168-1702(99)00023-4

viruses were carried out using permissive cell systems. However, it is also important to study the growth behavior of herpesviruses in various non-permissive or semi-permissive cell systems to understand and dissect the fine details of the stages of their viral growth cycles. There have been some examples of such research. Swine testis (ST) cells were abortive to herpes simplex virus (HSV) because the virus was unable to enter the cells through the plasma membrane (Subramanian et al., 1994, 1995). For ST cells, virus entry was critically reduced, DNA from the input virus could not be detected, and virus proteins were not synthesized. Murine resident peritoneal macrophages (ResPMO) produced viral immediate early (IE) protein in the course of HSV-1 infection, but early promoter activities were markedly reduced probably under the influence of infected cell-specific protein0 (ICP0) and therefore were not permissive to HSV (Leary et al., 1985; Tufaro et al., 1987; Sit et al., 1988; Mittel and Field, 1989; Morahan et al., 1989; Wu et al., 1993). Tufaro et al. (1987) isolated a mutant line of mouse L cells, in which the growth of HSV was abortive. HSV entered the mutant cells normally, negotiated the early stages of infection, yet were impaired at a late stage of virus maturation. It was concluded that the export of newly made glycoproteins was defective in the mutant cells. These studies indicated that the growth of herpesvirus could be arrested at various stages in different systems. Bovine herpesvirus-1 (BHV-1), which causes economically important diseases in the respiratory tract of cattle, belongs to the α-herpesvirus group (Armstrong et al., 1961; Roizman et al., 1981) but unlike other members of the group, such as HSV-1 or PRV, it has a relatively narrow host range in vitro. BHV-1 can replicate well in bovine-derived cell lines such as Madin Darby bovine kidney (MDBK) cells but grows poorly in rodent cell lines such as HmLu-1 cells derived from hamster lung. But little knowledge has been obtained about the host specificity. In this study, we investigated at which step BHV-1 replication was restricted in semi-permissive HmLu-1 cells.

2. Materials and methods

2.1. Viruses and cells

The virus strain used in this study was the BHV-1 Los Angeles (LA) strain re-cloned in our laboratory by limiting dilution. A recombinant BHV-1/RSV/p32 was obtained by inserting a 852 bp *Bam*HI fragment. This fragment contained the whole open reading frame of the major surface antigen p32 of *Theileria sergenti* (Matsuba et al., 1993) in the thymidine kinase gene of BHV-1 (LA) (Otsuka and Xuan, 1996). The p32 gene was flanked with the rous sarcoma virus long terminal repeat (LTR) at the 5′ side and with an SV40 poly A signal at the 3′ side (Sakai et al., 1990). MDBK and HmLu-1 (Kurogi et al., 1976) (hamster lung fibroblast, re-cloned in our laboratory) cells were cultured in Eagle's MEM (autoclavable, Nissui, Japan) containing heat inactivated 7.5% fetal calf serum (FCS) and 60 μg/ml kanamycin. The fetal calf serum was negative for BHV-1 antibody.

2.2. Infectious center assay

MDBK or HmLu-1 cells in 35 mm dishes were incubated with 1.0×10^5, 1.0×10^4 or 1.0×10^3 plaque forming unit (pfu) of BHV-1 at a multiplicity of infection (moi) of 0.1, 0.01 or 0.001, respectively, at 4°C for 2 h to allow the virus to be adsorbed into the cells. After the virus inoculum was removed, the monolayers were rinsed three times with 1 ml PBS, added to 1 ml medium and incubated at 37°C. At 0, 12, 24, 48, and 72 h post infection (p.i.) the cells were washed with PBS, treated with trypsin at 37°C for 10 min and collected by centrifugation at 3000 rpm for 5min. The cell pellets were washed three times with PBS, diluted with the medium and added to MDBK monolayers formed in 24 well plates. The cultures were incubated for 48 h, fixed and stained and the number of plaques was counted.

2.3. Viral DNA replication in infected cells

Confluent monolayers of MDBK or HmLu-1 cells in 60 mm dishes were infected with BHV-1(LA) at a moi of 5. At various times p.i., total

DNA (cellular and viral DNA) was extracted by the method described previously (Xuan et al., 1990). Briefly, infected cells were washed with PBS, lysed in an extraction buffer (0.1 M Tris–HCl (pH 9.0), 1% SDS, 0.1 M NaCl and 1m M EDTA), and then treated overnight with 1 mg/ml of pronase E. The lysates were extracted with phenol–chloroform, and the DNA was precipitated by ethanol. DNA samples (0.5 mg) were digested with *Eco*RI and subjected to 0.7% agarose gel electrophoresis in Tris–borate buffered solution (90 mM Tris–borate (pH 8), 2 mM EDTA, and 1 mg/ml ethidium bromide). After photographing on a UV light trans-illuminator, the agarose gel was soaked in 0.2 M HCl for 5 min, in 0.5 M NaOH containing 0.6 M NaCl for 45 min, and then neutralized in 1.0 M Tris–HCl (pH 7.0) buffer containing 0.6 M NaCl. The DNA fragments were transferred to a Biodyne nylon membrane B (PALL) by capillary action with 20 × SSC (3 M NaCl, 0.3 M trisodium citrate dihydrate). After air drying and baking at 80°C, the nylon membrane was hybridized with nick-translated probes in a buffer containing 50% formamide, 0.6 M NaCl, 0.2 M Tris–HCl (pH 8.0), 20 mM EDTA and 0.5% SDS at 42°C overnight. The membrane was then washed three times in the same buffer, dried and exposed to Fuji Medical X-ray film. A BHV-1 specific probe was prepared from the *Xho*I–*Bgl*II 1.7 kbp fragment in the open reading frame (orf) of the BHV-1 gC gene (Fitzpack et al., 1989). This probe was used to detect BHV-1 DNA.

2.4. RNA extraction and Northern blot hybridization analysis of IE genes

Confluent monolayers of MDBK or HmLu-1 cells in 75-cm^2 flasks were infected with BHV-1 at moi 5, and incubated at 37°C. At various times total RNA was extracted with guanidium thiocyanate (Chomczynski and Sacchi, 1987). Briefly, the cells were rinsed three times with ice-cold PBS and lysed with 2 ml denaturing solution D (50% guanidium thiocyanate, 10 mM Tris–HCl (pH 7.5), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The viscous lysate was forced to pass through a 23G needle several times to reduce viscosity and mixed with 0.2 ml 2 M sodium acetate, 2 ml water-saturated phenol, and 0.4 ml chloroform containing 2% isoamyl alcohol. The mixture was centrifuged at 10 000 × *g* for 20 min, and the RNA was precipitated from the supernatant with 50% isopropanol. The RNA was re-dissolved in 1 ml of the denaturing solution, re-precipitated with isopropanol and finally dissolved in a 10% MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer containing 50% formamide and 16% formaline. Pol A (+) mRNA was purified by Oligotex-dT30 (Takara; 9020), and 30% of each RNA sample was heated at 65°C for 15 min and separated in 1.0% agarose gel (1% agarose, 10% MOPS buffer) by electrophoresis in a running buffer (10% MOPS, 16.6% formalin) according to the method described earlier (Chomczynski and Sacchi, 1987). RNA was transferred to a Biodyne nylon membrane B by capillary action with 20 × SSC. After air drying and baking at 80°C, the membrane was hybridized with a DNA probe in a hybridization buffer (50% formamide, 0.6 M NaCl, 0.2 M Tris–HCl (pH 8.0), 20 mM EDTA, 0.5% SDS) at 42°C overnight. The membrane was then washed three times with the same buffer, dried and exposed to Fuji Medical X-ray film. The nick-translated 11 kbp linear DNA digested with *Eco*RI and *Hin*dIII (Wirth et al., 1992; Schwyzer et al., 1993) was used as a probe to detect transcripts of BICP4 and BICP0.

2.5. Antibodies against BICP4

The 7.6 kbp *Xho*I fragment of the BICP4 gene (Wirth et al., 1991; Schwyzer et al., 1993) was inserted at the SalI site of the pGEMEX2 plasmid (Promega) so that the coding region of the BICP4 gene was in frame. *Escherichia coli* JM109 (DE3), a host strain which contained an IPTG-inducible gene for T7 RNA polymerase, was transformed with the recombinant plasmid, and the BICP4 antigen was expressed as a T7 gene 10 fusion protein by incubating in L-broth containing 0.5 mM IPTG. The BICP4 antigen was extracted and precipitated according to the instruction in the Promega technical bulletin. Mice (ddy) were inoculated i.p. with the BICP4 antigen, once in Freund's complete adjuvant, and twice in Freund's

incomplete adjuvant at 7 day intervals. Blood was collected from the mice 7 days after the last inoculation, and the prepared sera were used for Western blot analysis.

2.6. Western blot analysis of BICP4

Confluent monolayers of MDBK or HmLu-1 cells in 60mm dishes were infected with BHV-1 (LA) at a moi of 5. After incubating over various intervals, the cells were washed with PBS, scraped in PBS, centrifuged and dissolved in 200 μl of a sample buffer (62.5 mM Tris–HCl (p H6.8), 2% SDS, 0.7 M mercapto-ethanol, 10% glycerol and 0.01% bromophenol blue). The samples were sonicated, heated in a boiling-water bath for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were electroblotted to a polyvinylidene difluoride paper (Millipore) as described previously (Towbin et al., 1979). The blotted paper was incubated at 37°C for 1 h with antibodies against BICP4. After washing with PBS, it was incubated with horseradish peroxidase labeled goat anti-mouse antibodies for 1 h, washed again with PBS and developed with PBS containing 0.5 mg/ml diaminobenzidine and 0.01% hydrogen peroxide until visible bands appeared. The reaction was stopped by rinsing the paper with PBS.

2.7. Preparation of radio-isotope labeled virus and adsorption test

Monolayers of MDBK cells grown in 100 mm dishes were infected with BHV-1 (LA) at moi 3. The infected cells were incubated in a methionine-free medium containing 100 μCi [^{35}S]methionine between 5 and 24 h p.i. Virions were collected from the medium at 24 h by centrifugation at $100\,000 \times g$ for 2 h in a Hitachi ultracentrifuge (type 55p-72) with a RPS40T rotor. The pellet was suspended in 1 ml PBS, layered onto a linear potassium tartrate gradient (10–40%) and centrifuged with the same rotor at $100\,000 \times g$ for 2 h. The virion band was collected by fractionation and stored at −80°C. Labeled BHV-1 (1 ml) was subjected to an attachment assay after diluting 1000-fold with Eagle's MEM.

Cells in 12 hole plates were infected with labeled BHV-1 at 4°C. At indicated times, cells were washed with PBS three times, lysed with 100 μl extraction buffer (0.1 M Tris–HCl (pH 9.0), 1% SDS, 0.1 M NaCl and 1 mM EDTA), put onto a glass filter and air dried completely. Each filter was then soaked in Econofluor2 (NEN) in a vial, and the radioactivity was assayed using a solvent partition method and presented as the net dpm of products formed per hour.

2.8. Quantification of penetrated and nuclear transported virion

The fractionation of cells was performed as follows. MDBK or HmLu-1 cells in 60 mm dishes were infected with BHV-1/RSV/p32 at 4°C for 1 h, washed three times with ice-cold PBS and incubated at 37°C in the medium containing 400 μg/ml phosphonoacetic acid (PAA). At various times, the cells were washed with PBS, trypsin solution added (0.8% trypsin (w/v), 0.05% EDTA (w/v) in PBS) and incubated at 37°C for 15 min. After the trypsin treatment, the cells were washed three times with PBS. DNA was extracted by the method described in Section 2.3. The isolation procedure for nuclei was described previously (Preston, 1979). Cells in 60 mm dishes were infected with BHV-1/RSV/p32 at moi 5 at 4°C for 1 h, washed three times with ice-cold PBS and incubated at 37°C with a medium containing PAA. At various times, the cells were washed with PBS, scraped into PBS and centrifuged at 5000 rpm for 3 min by a minifuge at 4°C. The cell pellets were suspended in 200 μl lysis buffer (10 mM Tris–HCl (pH 7.5), 2 mM$MgCl_2$, 10 mM NaCl, 5 mM 2-mercaptoethanol, 0.5% (v/v) Nonidet P-40) and homogenized with a dounce homogenizer until almost all the cells were broken and the nuclei released under microscope examination. Homogenates were centrifuged at $2000 \times g$ for 2 min, and the pellets were washed three times with lysis buffer and once with sucrose buffer (10 mM Tris–Hcl (pH 7.5), 2 mM $MgCl_2$, 10 mM NaCl, 0.32 M sucrose) at $2000 \times g$ for 4 min. DNA was extracted from the nuclear preparation by the method described above. All work was carried out on ice or at 4°C except where

described otherwise. After ethanol precipitation, each DNA sample was dissolved in 20 μl water and a 1 μl aliquot was subjected to quantitative competitive (QC)PCR reaction. The QCPCR reactions to measure the amount of BHV-1/RSV/p32 in infected cells or nuclei were designed based on the method described by Gariglio et al. (1997). The primers used to detect *T. sergenti* p32 gene were as follows; 5′-AAGCTCGACCTTTCCCAT-3′ and 5′-CAGGCGATGAGAAGAGC-3′. Using the p32 gene in plasmid or BHV-1/RSV/p32 as a template, these primers yielded a 517 bp fragment by PCR amplification. A mutant template plasmid pUCp32cdel1, which contained a deletion in the p32 gene, was constructed by cleaving the parental plasmid pUCp32 at the *Tth*III1 site and deleting some nucleotides with Exonuclease III (Takara) and mung bean nuclease (Takara) according to the manufacturer's instructions. Using pUCp32cdel1 as a template, a 350 bp fragment was amplified by PCR using the same pair of the primers. Known amounts of mutant templates and samples (1 μl) were added to each reaction tube with Taq DNA polymerase Recombinant (GIBCO BRL) and amplified according to the instructions using a GeneAmp PCR system 2400 (Perkin-Elmer). The conditions were as follows; incubation at 94°C for 2 min, 35 or 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a single extension at 72°C for 5 min. Amplified DNA fragments (517 and 350 bp) were electrophoretically separated in 1.5% agarose gel. The DNA bands were compared using Luminous Imager ver. 1 for Macintosh (AI-C). The equivalent points, where equal amounts of 517 and 350 bp DNAs were synthesized, were determined according to the standard linear regression curves.

3. Results

3.1. Replication and infectious center formation assay of BHV-1 in MDBK and HmLu-1 cells

Replication of BHV-1 in permissive MDBK and semi-permissive HmLu-1 was compared. While we could observe distinct cytopathic effects (CPE) in BHV-1 infected the MDBK cells within 6 h p.i., and the cells were completely rounded by 12 h p.i., little CPE were observed in the HmLu-1 cells at 24 h p.i. However at 48 or 72 h p.i., most of the HmLu-1 cells in the infected culture were rounded (data not shown). Fig. 1A shows one step and multi step growth curves for BHV-1 in the MDBK and HmLu-1 cells. When infected at a moi 5, the titer of BHV-1 in the MDBK cells started growing exponentially after 3 h, reached a plateau between 12 and 24 h and declined afterwards. In contrast, the titer in HmLu-1 increased very little up to 12 h and increased very slowly afterwards until it reached a plateau at around 48 h. The yield of infectious viruses in the HmLu-1 cells was two orders of magnitude lower than that in the MDBK cells. In the case of infection at a low moi, the difference in the growth in permissive and semi-permissive cells appeared to be greater. In the MDBK cells, BHV-1 replicated exponentially between 16–24 h p.i. reaching up to 10^7 PFU or more at around 48 h p.i. regardless of the initial input of the virus. However, in the HmLu-1 cells, the onset of the virus replication was delayed, starting around 24 h p.i., and the yield of infectious virus was three orders of magnitude lower than in the MDBK cells. When the input virus was less, the yield was much lower.

Additionally, we performed infectious center assays. Cell monolayers were infected with BHV-1 at a low moi at 4°C and then incubated at 37°C. After the incubation period, the cells were trypsinized, and the infectious centers were scored on permissive MDBK cells (Fig. 1B). Most of the infected MDBK cells (about 10^6) became infectious centers at 24 h p.i. In contrast only a small population of BHV-1 infected HmLu-1 cells were infectious centers at 24 h p.i. 2.1×10^4, 6.0×10^2 and 2.0×10^1 infectious centers were formed when infected with 10^5, 10^4 and10^3 pfu BHV-1, respectively.

3.2. Viral DNA replication in BHV-1 infected cells

In order to investigate whether replication of viral DNA was restricted in the HmLu-1 cells, viral and cellular DNA were extracted together

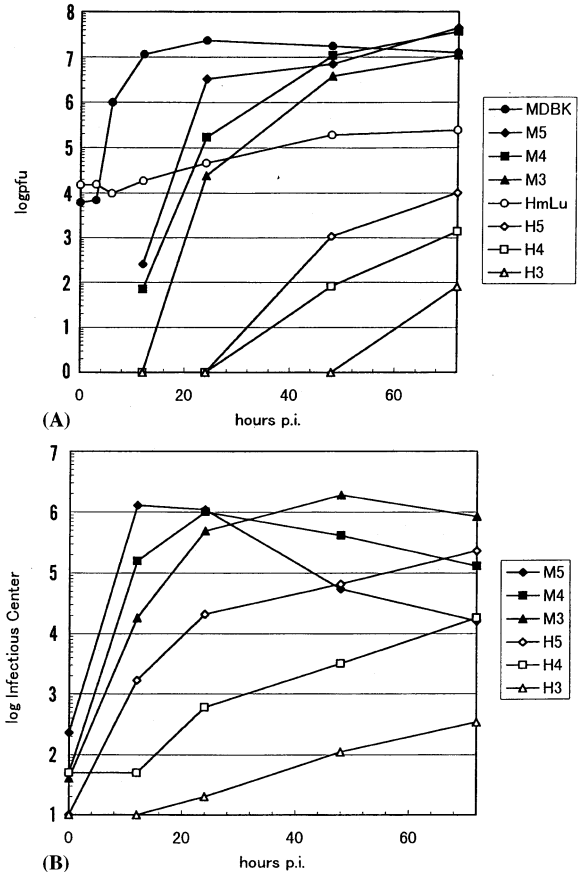

Fig. 1. Growth kinetics of BHV-1 in permissive MDBK and semi-permissive HmLu-1 cells. Monolayers of MDBK and HmLu-1 cells grown in 35 mm dishes were infected with 5.0×10^6 pfu (at an moi of 5, indicated as MDBK, HmLu), 1.0×10^5 pfu (moi = 0.1, M5 and H5), 1.0×10^4 pfu (moi = 0.01, M4 and H4), and 1.0×10^3pfu (moi = 0.001, M3 and H3) BHV-1. After 2 h adsorption, the media were removed, the cells were washed three times with PBS, and 1 ml fresh medium was added. At various times, both the cells and media were harvested and stored at −80°C. The samples were diluted in Eagle's MEM and seeded onto MDBK cells. The monolayers were overlaid with Eagle's MEM containing 0.5% methylcellurose. After 2 days incubation, the monolayers were fixed with ethanol, stained with 0.15% crystal violet solution, and plaque numbers were scored (A). The numbers of infectious centers were counted and shown in (B). The cells in the 35 mm dishes were attached with 1.0×10^5 (moi = 0.1, M5 and H5), 1.0×10^4 (moi = 0.01, M4 and H4), 1.0×10^3 (moi = 0.001, M3 and H3) pfu of BHV-1 at 4°C for 2 h. After the adsorption period, the virus inocula were removed, the cells were washed extensively and shifted to 37°C. At 0, 12, 24, 48, and 72 h p.i., the cells were trypsinized, washed three times with PBS and layered onto MDBK cells. The numbers of infectious centers were determined after crystal violet staining.

from BHV-1 infected cells and analyzed by Southern blot hybridization using the virus specific probe. Equal amounts of *Eco*RI-digested DNA samples were electrophoresed in an agarose gel and visualized with ethidium bromide (Fig. 2A). DNA bands in this gel were blotted, and hybridized with a nick translated probe containing the coding region of the BHV-1 gC gene. As shown in Fig. 2B, strong signals of BHV-1 DNA were observed in permissive MDBK cells starting at 6 h p.i., while no viral DNA was detected in semi-permissive HmLu-1 cells up to 12 h p.i., and very weak signals were seen at 24 and 48 h p.i. It would appear that only a very low level of replication of viral DNA took place in the HmLu-1 cells.

3.3. Expression of BICP4 in BHV-1 infected cells

Total cellular RNAs were isolated from BHV-1 infected MDBK and HmLu-1 cells. Equal amounts of RNA samples were separated by agarose gel electrophoresis, transferred to a membrane and hybridized to a nick translated probe. Schwyzer et al. (1993) and Wirth et al. (1991) reported the IE gene structure of BHV-1. We prepared the nick translated probe to detect both of the transcripts of the BICP4 and BICP0 genes according to their reports. As shown in Fig. 3, abundant transcripts of the BICP4 and BICP0 genes were detected in the MDBK cells at 3 and 6 h p.i., but only very low levels of these transcripts were found in the HmLu-1 cells until 12 h p.i. When RNA extracted from the MDBK cells at 6 h p.i. was diluted and compared with RNA from the HmLu-1 cells, it was found that the level of mRNA of BICP4 and BICP0 in the HmLu-1 cells was 0.1–0.01% of that in the MDBK cells (data not shown). Western blot hybridization analysis of BICP4 using mouse anti-serum revealed that the level of expression of BIE (200, 190, and 160 kDa) in the HmLu-1 cells was very low (Fig. 4), indicating that the rate limiting step of BHV-1 growth in the HmLu-1 cells was before the transcription of the IE genes.

3.4. Comparison of viral attachment

We compared the attachment and penetration of BHV-1 in permissive and semi-permissive cells

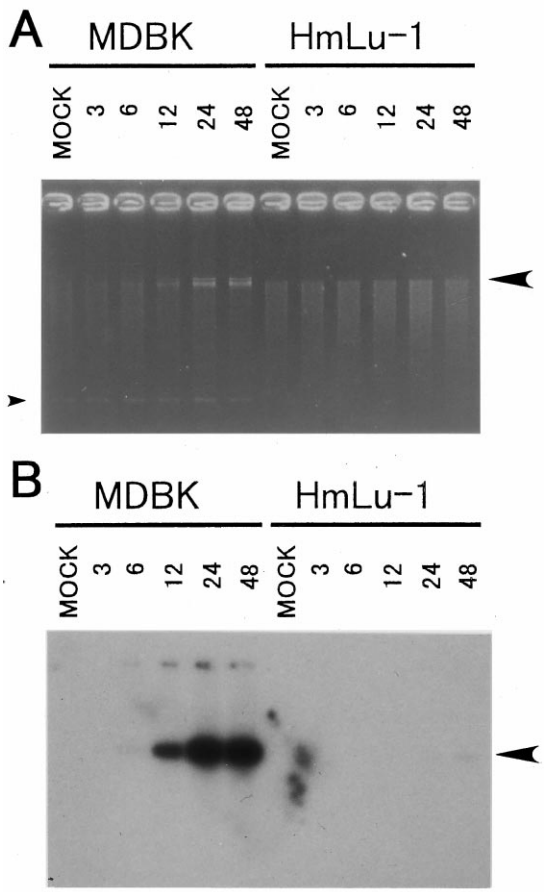

Fig. 2. Detection of BHV-1 DNA in permissive MDBK and semi-permissive HmLu-1 cells. Confluent monolayer cultures of MDBK cells or HmLu-1 cells were infected with BHV-1 at an moi of 5 and at 3, 6, 12, 24, and 48 h p.i., DNA was extracted as described in Section 2. Equal amounts were digested with EcoRI and electrophoresed in agarose gel. BHV-1 specific DNA fragments are depicted with a large arrow on the right side. Small fragments (small arrow indicated on the left side) should be MDBK cells specific DNA, not viral DNA (A). The DNA was then blotted and hybridized to a [^{32}P]-labeled nick-translated DNA probe which reacted to the BHV-1 specific gene. The arrow indicates BHV-1 specific bands (B).

using isotope labeled virions. The MDBK or HmLu-1 cells grown in 12 well plates were infected with [^{35}S]methionine labeled BHV-1 at 4°C. At 1, 2, and 4 h p.i., the cells were washed extensively and harvested, and the amounts of radioactivity associated with the cells were determined by a liquid scintillation counter. The results shown in Fig. 5A indicate that labeled BHV-1 was attached to both MDBK and HmLu-1 cells, but the amount of BHV-1 attached to HmLu-1 was about half of that attached to MDBK. BHV-1, as well as other herpesviruses, is known to bind to heparan sulfate on the cell surface, and this binding is blocked by heparin (Okazaki et al., 1991). When labeled BHV-1 was incubated at 4°C for 1 h in a medium containing 10 U/ml heparin and then added to the MDBK cells, the attachment of BHV-1 to the MDBK cells was reduced by about 60%, suggesting that about 40% of the labeled BHV-1 attached to the MDBK cells not via heparan sulfate but by other mechanisms. On the other hand, 90% of the adsorption of labeled BHV-1 to HmLu-1 was blocked by heparin treatment. This indicates that BHV-1 attached to the HmLu-1 cells mostly through heparan sulfate and only a small fraction of virus attached in a heparan sulfate independent manner.

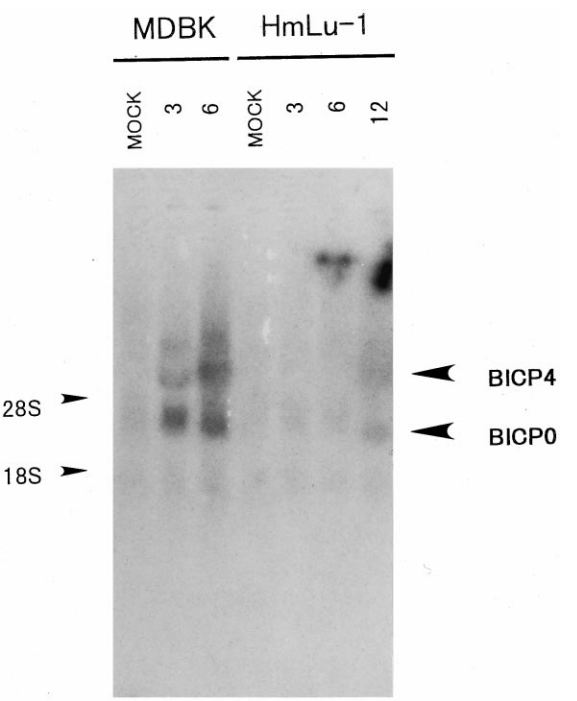

Fig. 3. Northern blot hybridization analysis of BICP4 and BICP0. The cells were infected with BHV-1 at an moi of 5. Total RNAs were extracted at the indicated times by means of the AGPC method, and equal amounts were subjected to agarose electrophoresis. Transcripts of BICP4 and BICP0 are indicated.

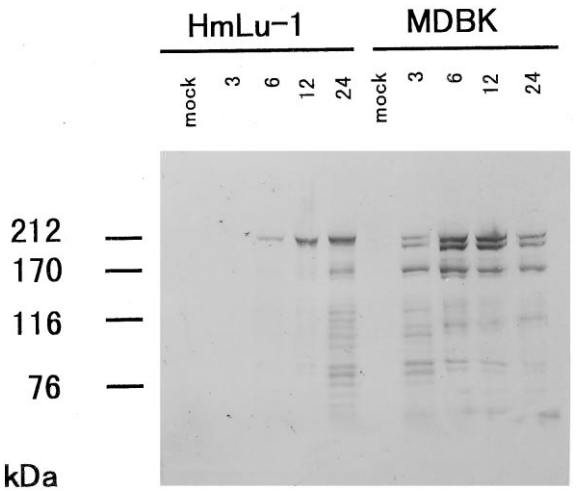

Fig. 4. Immunoblot hybridization analysis of BICP4, the major transcription regulatory protein, from BHV-1 infected HmLu-1 and MDBK cells. The cell proteins were harvested at 3, 6, 12, and 24 h p.i., and equal quantities were SDS-polyacrylamide gel electrophoresed and immunoblotted. Molecular weight markers are indicated on the left side.

The effect of heparin wash on BHV-1 which were already attached to the cells was studied. Monolayers were infected with [^{35}S]methionine labeled BHV-1 at 4°C. At 0, 1, and 2 h p.i., the cells were washed three times with PBS or incubated with heparin solution followed by washing twice with PBS. The cells were harvested, and the radioactivities associated with the cells were determined. As shown in Fig. 5B, once BHV-1 was attached onto either the MDBK or HmLu-1 cells, only 10% of the attached virions were released by washing with heparin. These results seemed to imply that immediately after the attachment via heparan sulfate molecules, BHV-1 virions became bound to the surface of the MDBK and HmLu-1 cells via non-heparan sulfate molecules.

3.5. Fusion of BHV-1 to the cell membrane

After attaching to the cell membrane, herpesvirus enters into the cells by pH-independent fusion between the viral envelope and the cell plasma membrane (Wittels and Spear, 1990). In order to measure the viral DNA that fused to the cells, QCPCR was used. In this series of experiments we used a BHV-1 recombinant, BHV-1/RSV/p32, which carries the p32 gene of *T. sergenti* in the BHV-1 genome, in place of wild type BHV-1. The reasons are as follows: (1) BHV-1 DNA is difficult to amplify by PCR due to a high GC content, (2) we established that the p32 gene can be easily amplified by PCR even it was integrated into BHV-1 DNA, and (3) the recombinant BHV-1/RSV/p32 behaved in the same way

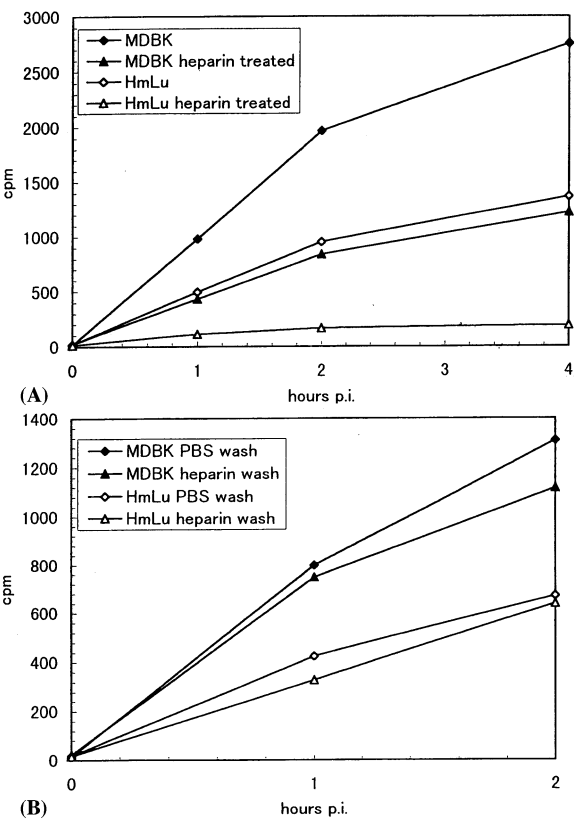

Fig. 5. Adosorption of radioisotope labeled BHV-1. MDBK and HmLu-1 cells were infected with [^{35}S]methionine labeled BHV-1 (MDBK, HmLu) or infected with labeled BHV-1 previously treated with heparin (MDBK heparin treated, HmLu heparin treated) at 4°C. At 0, 1, 2, and 4 h p.i., the cells were washed with PBS three times and collected, and the radioactivities associated with the cells were counted (A). The cells were infected with [^{35}S]methionine labeled BHV-1 at 4°C. At 0, 1, and 2, h p.i., the cells were washed with PBS (MDBK PBS wash, HmLu PBS wash) three times or inoculated with heparin (500 μg/ml in PBS) for 2 min followed by washing with PBS twice (MDBK heparin wash, HmLu heparin wash). The cells were collected and radioactivities associated with the cells were counted (B).

as the wild type BHV-1 in the MDBK or HmLu-1 cells. To quantitate viral DNA in the sample, a series of known amounts of a competitor template plasmid pUCp32cdel1 was mixed with the sample DNA, and the series of mixtures of the sample and the competitor were subjected to PCR amplification using the pair of primers described above. The PCR products were separated by 1.5% agarose gel electrophoresis as shown in Fig. 6A. BHV-1/RSV/p32 contains the wild type p32 gene which yields a 517 bp PCR product, and the competitor plasmid pUCp32cdel1 contains the p32 gene with a deletion which yields a 350 bp PCR product. When an equi-molar amount of BHV-1/RSV/p32 and the competitor plasmid were present in the sample mixture, the same amounts of the 517 bp product and the 350 bp product would be present in the PCR product. The amount of viral DNA in the samples was determined by estimating such a point by analyzing the data in Fig. 6A as described in Section 2. The MDBK or HmLu-1 cells were infected with BHV-1/RSV/p32 at 4°C for 1 h, washed extensively with cold PBS and incubated with medium at 37°C for 0, 1, 2, and 5 h. The virions attached onto the surface of cells during incubation at 4°C and penetrated into the cells when the temperature was shifted to 37°C (Huang and Wagner, 1964). To prevent the replication of viral DNA, 400 μg/ml phosphonoacetic acid (PAA) was included in the medium. Total DNA (cellular and viral DNA associated with cells) was extracted before and after trypsin treatment, and the amount of viral DNA was quantitated as above. The trypsin treatment removes attached virions from the cell surface but does not remove fused (penetrated) virions. The results are shown in Fig. 6B. After incubating at 4°C for 1 h, 1.02 pg equivalent (mean of three independent samples) BHV-1/RSV/p32 DNA was attached to the MDBK cells. However, trypsin treatment removed over 90% of the attached viral DNA, and only 0.095 pg equivalent viral DNA was found to be associated with the cells. The attached virions became more resistant to trypsin treatment when infected MDBK cells were incubated at 37°C; 0.37, 0.38, and 0.50 pg equivalent viral DNA were detected in the cells after 1, 2, and 5 h incubation periods, respectively. In the MDBK cells, therefore, about 49% of the attached virions became trypsin resistant after 5 h incubation at 37°C.

In semi-permissive HmLu-1 cells, QCPCR detected 0.42 pg equivalent of viral DNA that was attached to the cells after incubating at 4°C for 1 h. This indicated that BHV-1 attached HmLu-1 was about 40% of that attached to the MDBK cells, and this ratio was comparable to the isotope-labeled virus attachment experiments. After 1, 2, and 5 h incubation at 37°C, however, only 0.050, 0.093, and 0.01 pg equivalent DNA were detectable in trypsin treated HmLu-1 cells, respectively. The results indicated that after 2 h incubation at 37°C (3 h p.i.), the amount of virions which penetrated into the HmLu-1 cells was 24% of that which penetrated into the MDBK cells. It was also noted that the amount of trypsin resistant, cell associated virions in the HmLu-1 cells decreased after 5 h incubation while in the MDBK cells it increased.

It can be concluded that BHV-1 can attach to HmLu-1 cells, but the fusion and penetration process may be at a low level.

3.6. Nuclear transport of viral DNA

Additionally, we analyzed the nuclear transport of BHV-1 in permissive MDBK and semi-permissive HmLu-1 cells by QCPCR. After adsorption of BHV-1/RSV/p32 onto the cells for 1 h at 4°C, the cells were washed extensively with ice cold PBS and incubated with medium at 37°C. At 1, 3, 6, and 12 h p.i., the cells were washed with PBS, and the nuclei were isolated as described in Section 2. The DNA was extracted from the nuclear fraction, and the amount of viral DNA was quantitated by QCPCR. As shown in Fig. 6B, in permissive MDBK cells, 0.0100, 0.173, 0.364, and 0.116 pg equivalent DNA were transported to the nuclei at 1, 3, 6, and 12 h p.i., respectively. The amount of BHV-1 DNA in the nuclei of MDBK decreased at 12 h p.i. probably because the viral DNA was degraded when the viral DNA replication was inhibited by PAA. In the nuclei of HmLu-1, the amount of viral DNA was below the detection level of the method employed here ($<$ 0.001 pg equivalent).

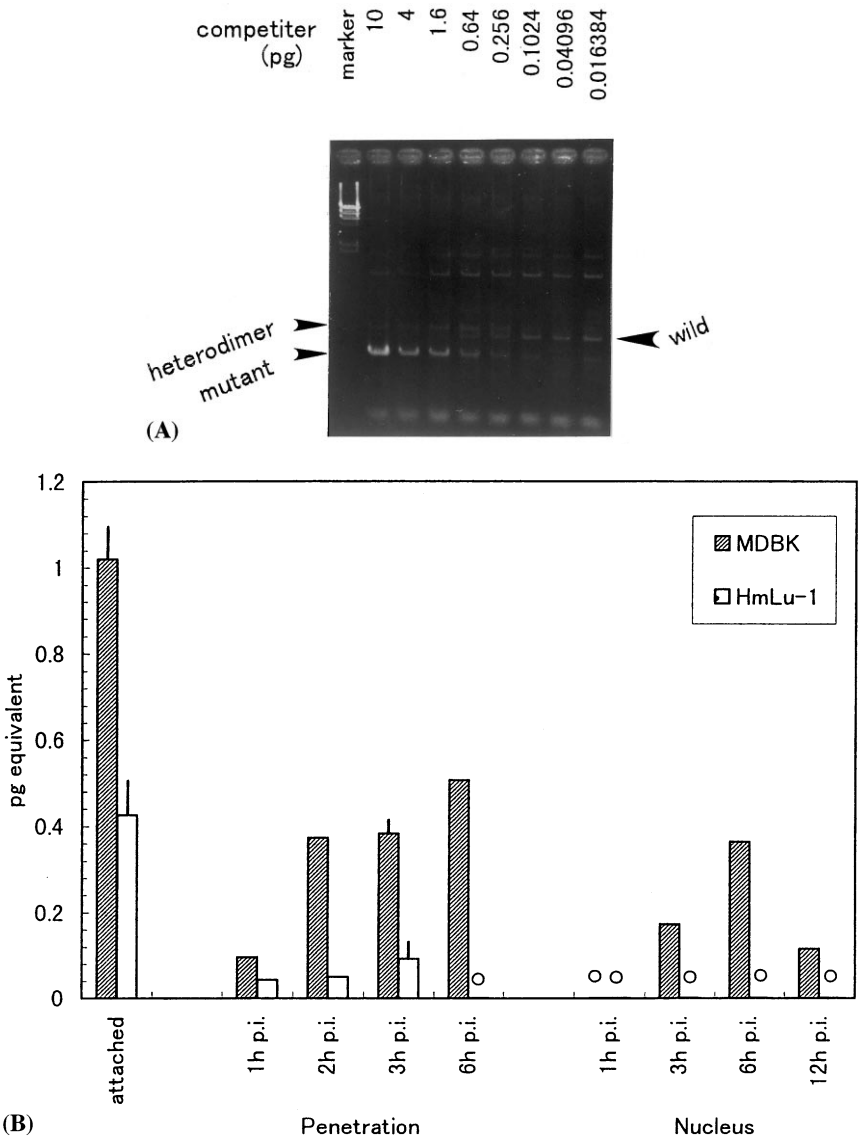

Fig. 6. Measurement of penetration and transportation to the nuclei by QCPCR of p32 gene integrated into BHV-1. Sample DNAs were co-amplified with a 2.5 times dilution series of competitor plasmid ranging from 10 pg (lane 1) to 0.016384 pg (lane 8). The amounts of wild-type p32 DNA were determined from linear regression curves against the amounts of mutant plasmid pUCp32cdel1 DNA. p32 DNA, namely BHV-1 DNA were indicated as pg equivalent of mutant plasmid pUCp32cdel1. A representative electrophoresis (MDBK, 6 h p.i., trypsin treated) is shown (A). (B) Shows the result of the quantitation of BHV/RSV/p32 genomes. The bar on the left side (shown as 'attached') indicates the attached virus on the MDBK and HmLu-1 cells during inoculation of 1 h at 4°C. After the attachment period, the cells were washed and the virus DNA associated with the cells were measured. The graph in the middle ('Penetration') indicates the trypsin resistant virus. After the attachment period, the cells were washed and temperature shifted to 37°C. At 1, 2, 3, and 6 h p.i. (0, 1, 2, and 5 h incubation at 37°C), the cells were washed, trypsinized, and the virus DNA associated with the cells were measured. The right graph is BHV-1 transported to the nucleus. After the attachment period, the cells were washed and temperature shifted to 37°C. DNA was extracted from the nucleus at 1, 3, 6, and 12 h p.i. (that was 0, 2, 5, and 11 h after adsorption), and the virus DNA were measured. Four experiments of penetration (3 h p.i.) and three experiments of attached were performed independently, and their S.D. were depicted. 'o' On the bars indicates that the quantities were less than 0.001 pg equivalent.

4. Discussion

Replication of the α-herpesvirus group, to which BHV-1 belongs, is well studied in permissive cell systems (Roizman and Sears (1990) and others). When permissive cells are infected with a virus, the following sequential events take place: (1) the virus particles attach onto the cell surface, (2) the viral envelope fuses to the cell plasma membrane and the virus capsid is released into the cytoplasm, (3) the released capsid is transported to the nucleus, (4) the IE class of the viral genes are expressed, (5) the IE products induce other classes (early, late) of viral genes, (6) the virus DNA replicats, (7) the newly synthesized virus DNA and proteins assemble automatically, (8) particles surrounded by envelopes bud from the cells.

In semi- or non-permissive systems, the growth of the virus is arrested at a point or points of the above mentioned steps. To study the growth behavior of the virus in a semi- or non-permissive system is very important because it allows for the analysis of the fine details of the particular step where the growth is arrested. In this study we investigated and elucidated the growth behavior of BHV-1 in semi-permissive HmLu-1 cells compared with that in permissive MDBK cells. The HmLu-1 cells have been used to propagate Akabane virus (Kurogi et al., 1976; Liao et al., 1996), bovine corona virus (Fukai et al., 1998), bluetongue virus (Ianconescu et al., 1996) and PRV (Shibata et al., 1991; Matsuda Tsuchida et al., 1992). However, BHV-1 grows poorly in HmLu-1 cells. When the HmLu-1 cells were infected with BHV-1 at low moi, very little CPE was observed, and the virus titer hardly increased after prolonged incubation periods. Infection at high moi caused distinct CPE, and the virus titer increased slightly (less than 1% of that in the MDBK cells). The timing of the virus replication was also delayed in the HmLu-1 cells. The transcripts of the BICP4 and BICP0 genes were synthesized in the MDBK cells immediately after infection by BHV-1, but only a very small quantity of both transcripts was found in the HmLu-1 cells at 12 h p.i. or later. It would appear that the cause of the poor growth in the HmLu-1 cells exist in the step or steps before the induction of the IE gene.

It has been reported earlier that α-herpesvirus adsorbed onto the cell surface in two distinct ways (McClain and Fuller, 1994). One adsorption mechanism is through the interaction between viral glycoproteins (gC and gB) and heparan sulfate on the cell surface. This adsorption mechanism is inhibited by heparin (WuDunn and Spear, 1989). The other adsorption mechanism, which is not inhibited by heparin, is not well understood (Lee and Fuller, 1993). Radioisotope labeled BHV-1 was adsorbed onto non-permissive HmLu-1 cells although the amount of adsorbed virus was about 50% of that adsorbed onto permissive MDBK cells. When the virus was treated with heparin before infection, the adsorption onto HmLu-1 cells was reduced by 90%. This suggests that 90% of BHV-1 was adsorbed onto semi-permissive HmLu-1 cells through heparan sulfate, and only 10% was adsorbed through the other mechanism. On the other hand, 60% of BHV-1 was adsorbed onto permissive MDBK cells through heparan sulfate and 40% through the heparan sulfate independent mechanism. Therefore, it would appear that the HmLu-1 cells are deficient in their ability to adsorb BHV-1 through the heparan sulfate independent mechanism. Because the heparin wash did not remove the virus which was already attached to either permissive MDBK or semi-permissive HmLu-1 cells, we concluded that after heparan sulfate binding, BHV-1 quickly bound to a cellular factor other than heparan sulfate and became resistant to the heparin wash.

Virus particles fused with the plasma membrane are resistant to trypsin treatment while particles which are just adsorbed on the cell surface are susceptible to trypsin. Quantitative analysis by QCPCR revealed that BHV-1 adsorbed to permissive MDBK cells penetrated into the cells, became trypsin resistant, and gradually were transported to the nuclei. On the other hand, the greater part of virus adsorbed to HmLu-1 remained trypsin sensitive after prolonged incubation and virus DNA was not detectable by the method employed in this study. However, a small number of BHV-1 virions must have reached the nuclei and replicated because a small fraction of BHV-1 infected HmLu-1 became infectious centers as shown in Fig. 1B.

Our results suggest that the major bottleneck for the growth of BHV-1 in the HmLu-1 cells may exist at the level of the penetration of the virion. Many viral and cellular factors are involved in the herpesvirus penetration which is probably a multi-step event. Montgomery et al. (1996) cloned herpesvirus entry mediator (HVEM/HveA) and demonstrated that HVEM mediated the entry of several wild type HSV strains into non-permissive CHO-K1 and ST cells. Mutations in the HSV envelope glycoprotein D (gD) significantly reduced HVEM-mediated entry, suggesting that HVEM functions via HSV gD (Montgomery et al., 1996). Other evidence existed that gD was important for fusion. Abortive cells could be made resistant to HSV entry while retaining the ability to bind a virus by transfection with plasmid expressing HSV gD (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989). Soluble forms of HSV gD inhibited virus entry into cells (Johnson et al., 1990). And recently, two other cell surface proteins named poliovirus receptor-related protein 1 (PRR1)/HveB and PRR2/HveC, were found to mediate HSV entry also by interaction with gD (Geraghty et al., 1998; Warner et al., 1998). For BHV-1, gD expressing cells were reported to resist BHV-1 penetration into the cells (Chase and Letchworth, 1994). Therefore it might be possible that HmLu-1 cells might lacked the HVEM like factor for BHV-1 gD while MDBK cells express such a factor allowing the penetration and the nuclear transport of BHV-1.

PRV grows well in HmLu-1 cells as well as in MDBK cells. We have constructed BHV-1 recombinants which express PRV glycoproteins (Otsuka and Xuan, 1996). In our preliminary experiments it was observed that the penetration into the HmLu-1 cells was greatly improved by BHV-1 recombinants which express PRV gB. Further analyses of these recombinants will shed some light on the question of virus–cell tropism.

Acknowledgements

T. sergenti p32 gene was provided by Dr M. Onuma (Hokkaido university, Hokkaido, Japan). This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

References

Armstrong, J.A., Pereira, H.G., Andrewes, C.H., 1961. Observations on the virus of infectious bovine rhinotracheitis virus, and its affinity with the herpesvirus group. Virology 14, 276–285.

Campadelli-Fiume, G., Arsenakis, M., Farabegoli, F., Roizman, B., 1988. Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J. Virol. 62, 159–167.

Chase, C.C.L., Letchworth III, G.J., 1994. Bovine herpesvirus 1 gIV-expressing cells resist virus penetration. J. Gen. Virol. 75, 177–181.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 163, 156–159.

Fitzpack, D.R., Babiuk, L.A., Zamb, T.J., 1989. Nucleotide sequence of bovine herpesvirus type 1 glycoprotein gIII, a structure model for gIII as a new member of the immunoglobulin superfamily, and implications for the homologous glycoproteins of other herpes viruses. Virology 173, 46–57.

Fukai, K., Sakai, T., Fujita, K., Abe, S., 1998. Evaluation of serum neutralizing antibodies to bovine coronavirus in cows and their calves using Hmlu-1 cells. Indian J. Med. Res. 108, 8–11.

Gariglio, M., Foresta, P., Sacchi, C., Lembo, M., Hertel, L., Landolfo, S., 1997. Suppression of high mobility group protein T160 expression impairs mouse cytomegarovirus replication. J. Gen. Virol. 78, 665–670.

Geraghty, R.J., Krummenacher, C., Eisenberg, R.J., Cohen, G.H., Spear, P.G., 1998. Entry of alphaherpesviruses mediated by poliovirus receptor related protein 1 and poliovirus receptor. Science 280, 1618–1620.

Huang, A.S., Wagner, R.R., 1964. Penetration of herpes simplex virus into human epidermoid cells. Proc. Soc. Exp. Biol. Med. 116, 863–869.

Ianconescu, M., Akita, G.Y., Osburn, B.I., 1996. Comparative susceptibility of a canine cell line and bluetongue virus susceptible cell lines to a bluetongue virus isolate pathogenic for dogs. In Vitro Cell. Dev. Biol. Anim. 32 (4), 249–254.

Johnson, D.C., Burke, R.L., Gregory, T., 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. 64, 2569–2576.

Johnson, R.M., Spear, P.G., 1989. Herpes simplex virus glycoprotein D mediated interference with herpes simplex virus infection. J. Virol. 63, 819–827.

Kurogi, H., Inaba, Y., Takahashi, E., et al., 1976. Epizootic congenital arthrogryposis-hydranencephaly syndrome in

cattle: isolation of Akabane virus from affected fetuses. Arch. Virol. 51, 67–74.

Leary, K., Connor, J.R., Morahan, P.S., 1985. Comparison of herpes simplex virus type 1 DNA replication and virus production in murine bone marrow-derived and resident peritoneal macrophages. J. Gen. Virol. 66, 1123–1129.

Lee, W.C., Fuller, A.O., 1993. Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not heparan sulfate. J. Virol. 67 (9), 5808–5897.

Liao, Y.K., Lu, Y.S., Goto, Y., Inaba, Y., 1996. The isolation of Akabane virus (Iriki strain) from calves in Taiwan. J. Basic Microbiol. 36, 33–39.

Matsuba, T., Kubota, H., Tanaka, M., et al., 1993. Analysis of mixed parasite populations of *Theileria sergenti* using cDNA probes encoding a major piroplasm surface protein. Parasitology 107 (4), 369–377.

Matsuda Tsuchida, A., Katayama, S., Okada, N., Okabe, T., Sasaki, N., 1992. Protection from pseudorabies virus challenge in mice by a combination of purified gII, gIII and gVI antigens. J. Vet. Med. Sci. 54, 447–452.

McClain, D.S., Fuller, A.O., 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology 198, 690–702.

Mittel, S.K., Field, H.J., 1989. Analysis of bovine herpesvirus type 1 thimidine kinase (tk) gene from wild-type virus and tk-deficient mutant. J. Gen. Virol. 70, 901–918.

Montgomery, R.I., Warner, M.S., Lum, B.J., Spear, P.G., 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87, 427–436.

Morahan, P.S., Mama, S., Anaraki, F., Leary, K., 1989. Molecular localization of abortive infection of resident peritoneal macrophages by herpes simplex virus type 1. J. Virol. 63, 2300–2307.

Okazaki, K., Matsuzaki, T., Sugahara, Y., et al., 1991. BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparin-like moiety on the cell surface. Virology 181, 666–670.

Otsuka, H., Xuan, X., 1996. Construction of bovine herpesvirus-1 (BHV-1) recombinants which express pseudorabies virus (PRV) glycoproteins gB, gC, gD and gE. Arch. Virol. 141, 57–71.

Preston, C.M., 1979. Abnormal properties of an immediate-early polypeptide in cells infected with herpes simplex virus type 1 mutant tsK. J. Virol. 32, 357–369.

Roizman, B., Carmichael, L.E., Deinhardt, F., et al., 1981. Herpesviridae definition, provisional nomenclature, and taxonomy. Intervirology 16, 201–217.

Roizman, B., Sears, A.A., 1990. In: Fields, B.N., Knipe, D.M., Chanock, R.M., et al. (Eds.), Fields Virology. Raven Press, New York, pp. 1795–1841.

Sakai, H., Siomi, H., Shida, H., Shibata, R., Kiyomasu, T., Adachi, A., 1990. Functional comparison of transactivation by human retrovirus rev nd rex genes. J. Virol. 64, 5833–5839.

Schwyzer, M., Vleck, C., Menekse, O., Fraefel, C., Paces, V., 1993. Promoter, spliced leader, and coding sequence for BICP4, the largest of the immediate-early proteins of bovine herpesvirus 1. Virology 197, 349–357.

Shibata, I., Inaba, Y., Akashi, H., 1991. Avirulent ts and thymidine kinase-deficient mutant of Aujeszky's disease virus. J. Vet. Med. Sci. 53, 663–670.

Sit, M.F., Tenney, D.J., Rothstein, J.L., Morahan, P.S., 1988. Effect of macrophage activation on resistance of mouse peritoneal macrophages to infection with heroes simplex virus type 1 and 2. J. Gen. Virol. 69, 1999–2010.

Subramanian, G., McClain, D.S., Perez, A., Fuller, A.O., 1994. Swine testis cells contain functional heparan sulfate but are defective in entry of herpes simplex virus. J. Virol. 68, 5667–5676.

Subramanian, G., LeBlanc, R.A., Wardley, R.C., Fuller, A.O., 1995. Defective entry of herpes simplex virus type 1 and 2 into porcine cells and lack of infection in infant pigs indicate species tropism. J. Gen. Virol. 76, 2375–2379.

Towbin, H., Steaehelin, T., Gordon, J., 1979. Electro-phoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Tufaro, F., Snider, M.D., McNight, S.L., 1987. Identification and characterization of a mouse cell mutant defective in the intracelluler transport of glycoproteins. J. Cell Biol. 105, 647–658.

Warner, M.S., Martinez, W., Geraghty, R.J. et al. 1998. A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by herpes simplex virus type 2, mutants of herpes simplex virus type 1 and pseudorabies virus. Virology (in press).

Wirth, U.V., Vogt, B., Schwyzer, M., 1991. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. J. Virol. 65, 195–205.

Wirth, U.V., Fraefel, C., Vogt, B., Vlcek, C., Paces, V., Schwyzer, M., 1992. Immediate-early RNA 2.9 and early 2.6 of bovine herpesvirus 1 are 3′ coterminal and encode a putative zinc finger transactivator protein. J. Virol. 66, 2722–2763.

Wittels, M., Spear, G., 1990. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus Res. 18, 271–290.

Wu, L., Morahan, P.S., Leary, K., 1993. Regulation of herpes simplex virus type 1 gene expression in nonpermissive murine resident peritoneal macrophages. J. Leukoc. Biol. 53, 61–65.

WuDunn, D., Spear, P.G., 1989. Initial interaction of herpes simplexvirus with cells is binding to heparan sulfate. J. Virol. 63 (1), 52–58.

Xuan, X., Horimoto, T., Ono, M., et al., 1990. Restriction endonuclease analysis of canine herpesviruses isolated in Japan. Jpn. J. Vet. Sci. 52 (6), 1181–1188.