

## Equine herpesvirus type 1 tegument protein VP22 is not essential for pathogenicity in a hamster model, but is required for efficient viral growth in cultured cells

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**ABSTRACT.** VP22 is a major tegument protein of Equine herpesvirus type 1 (EHV-1) that is a conserved protein among alphaherpesviruses. However, the roles of VP22 differ among each virus, and the roles of EHV-1 VP22 are still unclear. Here, we constructed an EHV-1 VP22 deletion mutant and a revertant virus to clarify the role of VP22. We found that EHV-1 VP22 was required for efficient viral growth in cultured cells, but not for virulence in a hamster model.

**KEY WORDS:** equine herpesvirus, tegument protein, VP22

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Equine herpesvirus type 1 (EHV-1: family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*) is a major cause of abortion in pregnant mares, respiratory infection in young horses and neurological diseases in horses of all ages [12]. The herpesvirus virion is composed of four concentric compartments including a linear double-stranded DNA, the capsid, the tegument and the envelope [16]. The tegument proteins of alphaherpesviruses, which are encoded by at least 15 viral genes, consist of the amorphous region between the nucleocapsid and the envelope [13].

EHV-1 VP22 (EVP22) is a tegument protein composed of 304 amino acids (aa) encoded by ORF11 [1]. VP22 is conserved among alphaherpesvirinae, but not among beta and gammaherpesvirinae [9]. HSV-1 VP22 (HVP22) is encoded by the UL49 gene [5], and BHV-1 VP22 (BVP22) is encoded by the UL49 gene [11]. Some alphaherpesviruses require VP22 homologs for viral replication, and others do not. For example, the VP22s of Marek's disease virus serotype 1 (MDV-1) and varicella-zoster virus (VZV) are essential for viral replication in cell culture [2, 3]. On the other hand, BVP22 and HVP22 are not essential for viral replication in cell culture, but they were shown to increase the pathogenicity in a natural host (BVP22) and in an animal model (HVP22) [4, 10, 11, 15], respectively. However, it remains unclear whether EHV-1 needs VP22 for viral replication. We previously reported the intracellular localization of EVP22 [14], although its functions are unclear.

In the present study, we constructed a VP22 deletion mutant and a revertant virus to clarify the role of VP22. The re-

sults suggest that VP22 is required for efficient viral growth in cultured cells, but not for virulence in a hamster model.

An EHV-1 bacterial artificial chromosome (BAC) clone, pAb4pBAC [8], was used to construct an Ab4p VP22 deletion mutant BAC clone (pAb4pΔVP22) and a revertant BAC clone (pAb4pΔVP22R) (Fig. 1). pAb4pΔVP22 was constructed by replacing the VP22 sequence of pAb4pBAC with an rpsL-neo cassette, and pAb4pΔVP22R was constructed by replacing the rpsL-neo cassette of pAb4pΔVP22 with the native VP22 sequence using the Ab4p genome as a template. Counter-selection BAC modification by Red/ET recombination system (Gene Bridges GmbH, Heidelberg, Germany) was used to construct pAb4pΔVP22 and pAb4pΔVP22R as described in the manufacturer's manual (Gene Bridges, version 3.0).

The rpsL-neo cassette (rpsL-neo gene) for replacing VP22 sequence was prepared as follows: A pair of primers was designed to amplify the insertion fragment using rpsL-neo template DNA (Gene Bridges GmbH) as the template. The forward primer was 5'-TAC AGC GCT AGT ATT AGA GTT TTG TAA GAG TTT ATT ATT AGC AAG TGA ATG GCC TGG TGA TGA TGG CGG GAT CG-3', and the reverse primer was 5'-GAG GCA CAT TTT ATT GAG GGC ACA GTG TTA TGA ATT TAT GCA AAT AAG CGT CAG AAG AAC TCG TCA AGA AGG CG-3'. Both primers consisted of 50-nucleotide homology arms and 24 nucleotides (underlined) for amplifying the rpsL-neo cassette sequence. A VP22 sequence with homology arms for constructing Ab4pΔVP22R was amplified by PCR with a pair of primers 5'-AAT CGT GAC GCT GGA GAT GTT G-3' and 5'-CTC GCA GGT GTC ATT ATA GC -3'.

pAb4p attB was constructed from Ab4p BAC. It had the attB sequence between ORF2 and ORF3 as a result of removing the BAC sequence with the Gateway® LR clonase reaction. Because Ab4p attB can be regarded as equivalent to the wild-type Ab4p [8], it was used as a parent strain of Ab4pΔVP22 and Ab4pΔVP22R. Infectious Ab4p attB,

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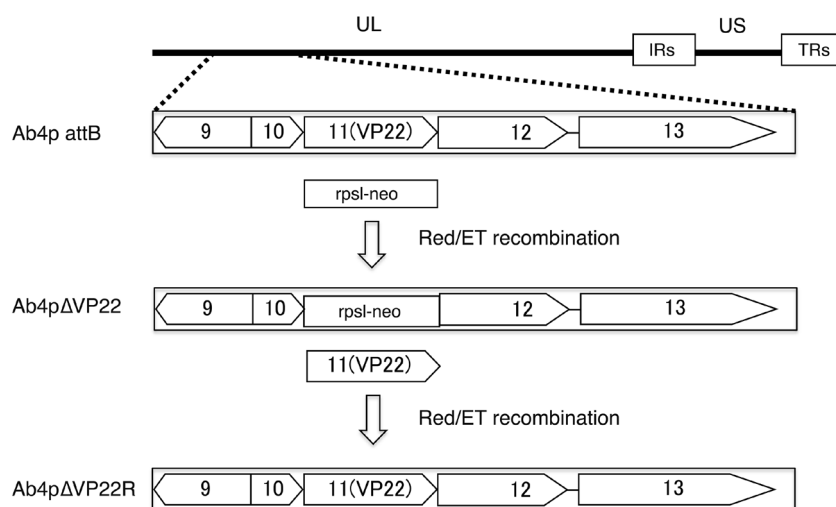


Fig. 1. Schematic diagram of the genome structure of mutant viruses and location of viral genes. UL, unique long; US, unique short; IR, internal repeat; TR, terminal repeat.

Ab4pΔVP22 and Ab4pΔVP22R viruses were generated as described previously [8].

To investigate the influence of rpsL-neo cassette insertion on transcription of the viral genes, transcript levels of viral genes next to rpsL-neo cassette including ORF10 (glycoprotein N) and ORF12 (VP16) at 8 hr post infection (hr.p.i.) were estimated by real time quantitative RT-PCR. Similar transcription levels of ORF10 and ORF12 were observed in cells infected with the deletion mutant of Ab4pΔVP22, the revertant of Ab4pΔVP22R or the parent of Ab4p attB (data not shown). RT-PCR analyses of ORF10 and ORF12 confirmed that the replacement of ORF11 with rpsL-neo cassette did not affect gene expression of genes next to rpsL-neo cassette in infected cells.

The growths of the viruses were first compared in one-step growth experiments in Madin-Darby bovine kidney (MDBK) cells growing in Minimum Essential Medium  $\alpha$  (MEM  $\alpha$ ; Wako, Osaka, Japan) supplemented with 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, U.S.A.). Monolayers of MDBK cells prepared in 24-well plates were inoculated with the viruses at an m.o.i. of 5 plaque-forming units (pfu)/cell. After 1 hr adsorption, cells were washed three times with MEM  $\alpha$  and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere in 1 ml/well of MEM. Supernatants and infected cells were collected at 0, 6, 9, 12, 24 and 48 hr.p.i. Collected samples were lysed by freezing and thawing three times to release cell-associated viruses, and supernatants were used as samples after centrifugation under the condition of 2,500 rpm for 5 min at 4°C. MDBK cells were inoculated with 10-fold dilutions of the samples and overlaid with 1.5% methylcellulose. After two days, viral titers were determined by counting the number of plaques using microscopy. Each experiment was conducted three times. The growth curves of Ab4p attB and Ab4pΔVP22R were similar, whereas the growth curve of Ab4pΔVP22 was significantly lower (Fig. 2). This suggests that VP22 is not essential for viral growth

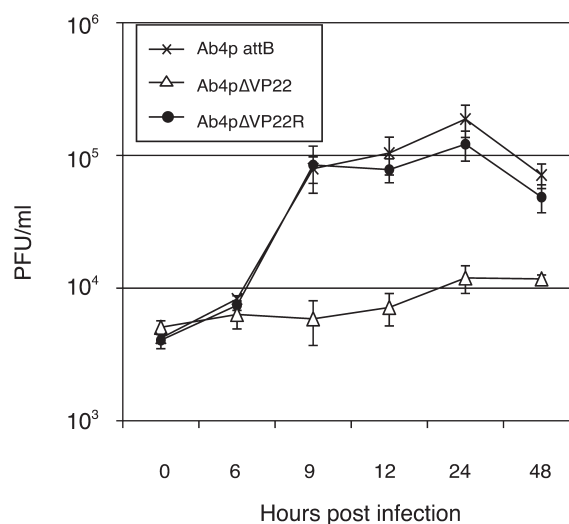


Fig. 2. One-step growth curves of Ab4p attB, Ab4pΔVP22 and Ab4pΔVP22R. MDBK cells were infected at an m.o.i. of 5 pfu/cell with each virus, and total viruses were collected at the indicated times. The titers of each sample were determined by plaque assay. Error bars indicate standard errors.

and is required for efficient viral growth in cultured cells.

We next conducted multi-step growth experiment and measured average plaque area to evaluate the efficiencies of the cell-to-cell spread. For the multi-step growth experiments, monolayers of MDBK cells prepared in 24-well plates were inoculated with each virus at an m.o.i. of 0.01 pfu/cell. After 1 hr adsorption, cells were washed three times with MEM  $\alpha$  and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere in 1 ml/well of MEM  $\alpha$ . Supernatants were collected as extracellular samples, and infected cells were collected in 1 ml/sample of MEM  $\alpha$  as intracellular samples after washing cells

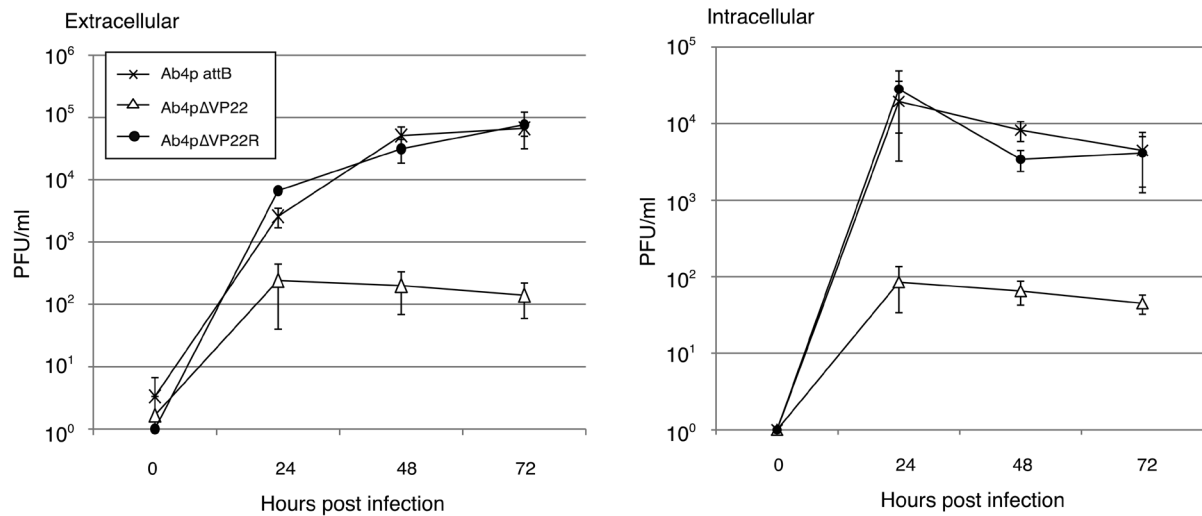


Fig. 3. Multi-step growth curves of Ab4p attB, Ab4pΔVP22 and Ab4pΔVP22R. MDBK cells were infected at an m.o.i. of 0.01 pfu/cell with each virus, and extracellular and intracellular viruses were collected at the indicated times. The titers of each sample were determined by plaque assay. Error bars indicate standard errors.

with MEM $\alpha$  three times at 0, 24, 48 and 72 hr.p.i. Infected cells were lysed by freezing and thawing three times to release cell-associated viruses, and supernatants were used as samples after centrifugation under the condition of 2,500 rpm for 5 min at 4°C. The virus titers were determined as described above. For the average plaque area measurement, the viruses were plated on MDBK cells and incubated for 3 days of incubation at 37°C under a 1.5% methylcellulose overlay. For each virus, plaque areas of 20–30 plaques for each experiment were determined in triplicate using ImageJ 1.42q software (<http://rsb.info.nih.gov/ij/index.html>). The growth curves of Ab4p attB and Ab4pΔVP22R were similar, whereas the growth curve of Ab4pΔVP22 was significantly lower (Fig. 3). The average plaque areas were determined from three experiments and were compared with Mann-Whitney *U* test. The average plaque area of Ab4pΔVP22 was smaller than the average plaque areas of Ab4p attB and Ab4pΔVP22R ( $P < 0.05$ ) (Fig. 4). The results of the multi-step growth experiment and average plaque area measurement suggest that EVP22 is associated with viral multiplication and plaque formation.

To evaluate the virulence of Ab4pΔVP22 *in vivo*, we used Syrian hamsters which require a lower concentration of EHV-1 than mice to show neurological symptoms [7]. The experiment was conducted on three-week-old specific pathogen-free (SPF) males (SLC, Hamamatsu, Japan) as described previously [7]. Hamsters were grouped into 4 groups of 4 animals per group, anesthetized with ether and were intranasally inoculated with 50  $\mu$ l of MEM containing  $3 \times 10^3$  pfu of virus per animal. A mock-infected group was inoculated with 50  $\mu$ l of MEM. The body weight of each animal was monitored every day for two weeks, and Mann-Whitney *U* test was used to determine the statistical significances. The animal experiments were certified (certification number 13045) and conducted under the guidelines of the Commit-

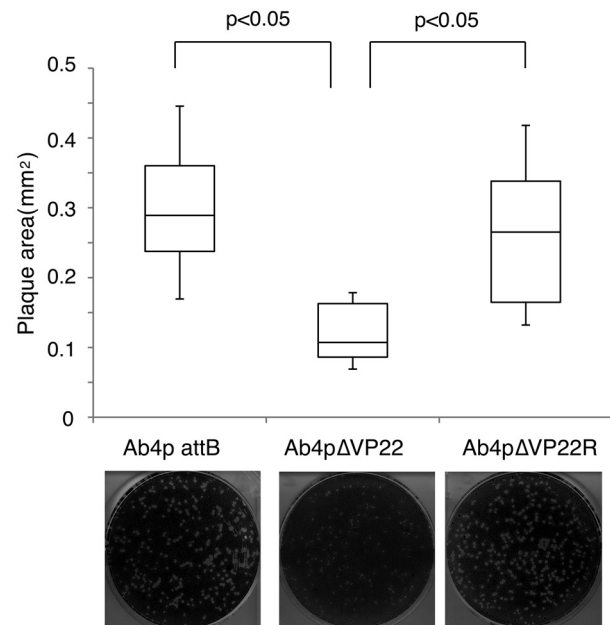


Fig. 4. Relative plaque areas of Ab4p attB, Ab4pΔVP22 and Ab4pΔVP22R in MDBK cells. Cells were seeded in 6-well plates and infected with each virus. At 3 d.p.i., cells were stained with crystal violet, and the sizes of 20–30 randomly-selected plaques of each virus were determined. The bars indicate the 90th, 75th, 50th, 25th and 10th percentiles from the top to the bottom.

tee of Animal Care and Welfare, Faculty of Applied Biological Science, Gifu University. The mean body weight changes and the numbers of dead hamsters are shown in Fig. 5 and Table 1, respectively. The mean body weights of hamsters inoculated with Ab4pΔVP22 were larger than that of Ab4p

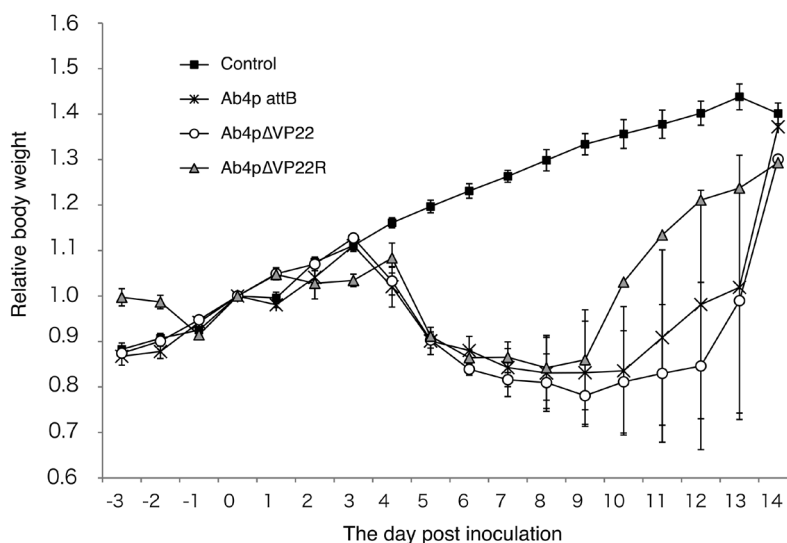


Fig. 5. Body weight changes of hamsters. Four groups of hamsters were infected with Ab4p attB, Ab4pΔVP22 and Ab4pΔVP22R at a dose of  $3 \times 10^3$  pfu/head, and MEM was inoculated to hamsters for a control group at day 0. Mean body weights of each group are expressed relative to the body weight of those measured on the day of infection (day 0). Error bars indicate standard errors. After day 9, some standard errors were not determined, because the numbers of hamster were too low.

Table 1. The numbers of died hamsters

| Viruses    | The day post infection |   |   |   |   |   |   |   |   |   |    |    |    |    |    |
|------------|------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|
|            | 0                      | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Ab4pΔVP22  | 0                      | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 1  | 1  |
| Ab4pΔVP22R | 0                      | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1  | 0  | 0  | 0  | 0  |
| Ab4p attB  | 0                      | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 1  | 0  | 1  |
| Control    | 0                      | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0  | 0  | 0  | 0  | 0  |

attB at 1-day post inoculation (d.p.i.) and Ab4pΔVP22R at 3 d.p.i. ( $P < 0.05$ ). However, the other body weight changes of each group were not significantly different (Fig. 5), and the numbers of died hamsters were not different among each group (Table 1), which suggests that the pathogenicities of the three viruses were not different and indicates that VP22 is not associated with the pathogenicity of EHV-1 in the hamster model.

Our results suggest that VP22 is required for efficient viral growth in cultured cells, whereas VP22 is not essential for pathogenicity in the hamster model. These data suggest that viral growth is not necessarily correlated with the pathogenicity in the hamster model of EHV-1 infection. However, because EHV-1 Ab4p is a highly pathogenic strain [6, 17], it is possible that the different result might be observed in animal experiments using other lower pathogenic strains. On the other hand, the VP22s of MDV-1 and VZV, which are in the same alphaherpesvirus family as EHV-1, were found to be essential for viral growth in cultured cells [2, 3]. In addition, BVP22 and HVP22 were reported to be associated with virulence in a natural host of BHV-1 and in an animal model of HSV-1 infection [4, 10, 11, 15], respectively. The finding

that the pathogenicity of Ab4pΔVP22 does not depend on its growth ability suggests that different alphaherpesviruses have different mechanisms of pathogenicity. These data indicate that virological function of EVP22 is not necessarily the same as that of other herpesviruses including MDV-1, VZV, BHV-1 and HSV-1. Therefore, the role of EVP22 might be different from those of other herpesviruses.

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