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Full Length Research Paper

Characterization of pseudorabies viruses produced in mammalian cells by viral genome transfection

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The full-length genome DNA of pseudorabies virus (PrV) was transfected into swine testis (ST) cells using calcium phosphate method to generate the infectious viruses. The resulting viruses were identified by indirect immunofluorescence and were used to infect African green monkey kidney (Vero) cells. Our results showed that the produced viruses had similar plaque morphology and growth properties with PrV vaccine strain, Bartha-K61. This method may produce PrV at an inexpensive cost.

Key words: Pseudorabies virus, transfection, virus plaque, cytopathic effect.

INTRODUCTION

Pseudorabies virus (PrV) is a swine neurotropic herpesvirus and it has common genome arrangements and many colinear genes with herpes simplex type 1 virus (HSV-1). Within the alphaherpesviruses, four genera have been established on the basis of genome sequence similarity (Minson et al., 2000; Klupp et al., 2004). They are the genera Varicellovirus, Simplexvirus, infectious laryngotracheitis-like viruses and Marek's disease-like viruses. Presently, PrV is grouped in the Varicellovirus genus (Klupp et al., 2004). PrV infection can result in Aujeszky's disease (AD) in swine and cattle. The AD has been one of the list B diseases of the Office international des épizooties (OIE). In addition to pig, PrV has a broad host range and can infect most mammals and some avian species (Tirabassi and Enquist, 2000). In young piglets and other susceptible species, PrV infection is often lethal and infected animals may die from central nervous system disorders (Klupp et al., 2004).

PrV genome is linear, double-stranded and noncircularly permuted DNA molecules (Lu et al., 1989). Due to its similar genome organization and infection characteristics with other herpesvirus including HSV-1, PrV has proven to be an excellent model system for alphaherpesvirus biology (Mettenleiter, 1994, 2000, 2002). The exploration on molecular biology of pseudorabies virus has significant impact on neurovirology and veterinary medicine (Pomeranz et al., 2005).

Although higher primates including humans are not susceptible to PrV infection, its potential transmission risk

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Abbreviations: PrV, Pseudorabies virus; **ST,** swine testis; **HSV-1,** herpes simplex type 1 virus; **AD,** Aujeszky's diseases; **EMEM,** Eagles' minimal essential medium; **DMEM,** Dulbecco´s modified Eagle's medium; **NBS,** newborn bovine serum; **PBS,** phosphate-buffered saline; **BBS,** borate buffered saline; **MOI,** multiplicity of infection; **p.i,** post infection; **CPE,** cytopathic effect; **PEDV,** porcine epidemic diarrhea virus.

and broad-spectrum host range of infection have limited the direct delivery of virus in/between different regions without any protection and prevention measures. However, due to the difference in viral gene sequence and infectivity, the accessibility of some given PrV isolates is a necessary prerequisite for studying the biological functions of PrV. In this study, the full-length genome of PrV strain Kaplan was transfected into mammalian cells by calcium phosphate method. The infectious viruses were produced and partially characterized. These data show that calcium phosphate transfection may be an alternative method to produce PrV in cells. For safety reason, it is better to transport PrV DNA genome between geographically differrent laboratories when PrV is required for biological research.

MATERIALS AND METHODS

Viruses and cells

PrV strain Kaplan and PrV vaccine strain, Bartha K-61 were propagated in African green monkey kidney (Vero) cells. Swine testis (ST) cells and Vero cells were maintained in Eagles' minimal essential medium (EMEM) and Dulbecco´s modified Eagle's medium (DMEM), respectively. Both were supplemented with 5% newborn bovine serum (NBS, Excell Bio. Shanghai, China) and 5% CO_2 in air and the cells were approximately passaged twice a week.

Transfection of PrV genome

The DNA of PrV Kaplan was isolated from purified PrV virions by phenol in the presence of sodium lauroylsarcosinate (Lomniczi et al., 1984). The extracted DNA purified with a DNA purified kit (KeyGen Biotech, Nanjing, China) was used to generate PrV by calcium phosphate transfection technique used. Briefly, 80 - 90% confluent ST cells grown on 6-well-plate were washed with phosphate-buffered saline (PBS) and then cultured with 2 ml EMEM medium without antibiotics in each well for 1 h. Meanwhile, 4 µg full-length PrV genome was gently mixed with 100 µl calcium phosphate solution and then the DNA-calcium phosphate mixture was added into 100 µl borate buffered saline (BBS) a (Beyotime, China) and incubated at room temperature (RT) for 20 min. The resulting mixture was added evenly into the above-mentioned ST cell monolayers and the transfected cells were incubated at 37°C with 5% CO_2. At the same time, calcium phosphate-BBS buffer mixture applied to cell monolayer was used as control. After approximately 8 h post-transfection, the cells were gently shaken and the suspended precipitations were discarded and replaced by 2 ml corresponding medium. At 36 h post transfection, the cell supernatant was har-vested for virus passage.

Passage of produced PrV

The cell supernatant was harvested and cell debris was removed by low-speed centrifugation after freezing and thawing three times. The virus particles-containing supernatant was used to inoculate ST cells for virus passage and cytopathic effect examination. The viruses were tittered using plaque assays as previously described (Li et al., 2009; Sui et al., 2010; Ren et al., 2010; Ren et al., 2011).

Confirmation of PrV infection by indirect immunofluorescence

To confirm the production of PrV, a mouse-derived polyclonal antibody derived from PrV strain Bartha K-61-immunized mice was used as primary antibody in indirect immunofluorescence analysis according to references with minor modifications (Liu et al., 2009; Schwegmann-Wessels et al., 2009; Meng et al., 2010). The ST cells were seeded on coverslides in 24-well plates and cultured in EMEM containing 5% NBS. The confluent cell monolayers were infected with the produced PrV strain Kaplan at a multiplicity of infection (MOI) of 10 for approximately 36 h. The cells were fixed with 3% (w/v) paraformaldehyde in PBS for 20 min, quenched in 0.1 M glycine for 5 min and washed with PBS. The cells were incubated with the primary antibody diluted in 1% bovine serum albumin (BSA) for 1 h followed by incubation with the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Zhongshan, China) for another 1 h in the dark. The cells were mounted on glass slides and examined under a fluorescence microscope.

Cytopathic effect (CPE) of Vero cells infected by PrV

To analyze the CPE of PrV-infected cells, Vero cells were maintained in DMEM containing 5% NBS. The cells were inoculated with the PrV at an MOI of 10. At 1 h post infection (h.p.i.), the medium was replaced with 1% methylcellulose-containing medium. At 36 - 48 h.p.i., the CPE caused by PrV was examined by light microscopy.

Growth kinetics of the virus

To analyze the property of PrV propagation, the single-step growth kinetics of the PrV Kaplan produced by genome transfection and PrV Bartha K-61 were compared according to published data (Whealy et al., 1988, 1992). Briefly, at 1 h.p.i., the inoculum at an MOI of 10 was removed from the Vero cells in 24-well plates, and the cells were treated with 40 mM citric acid, 10 mM KCl and 135 mM NaCl for 1 min and replaced with DMEM containing 2% NBS and antibiotics. At different time points (6, 12, 18, 24, 30, 36 and 42 h.p.i.), the virus-containing supernatant from the cells was harvested, and used to re-infect Vero cells in 24-well plates and the viral titer was determined by plaque assays as previously described (Ren et al., 2008; Sui et al., 2010; Li et al., 2010).

RESULTS

Production and confirmation of Pr*V*

The full-length genome of PrV was used to transfect ST cells via calcium phosphate method. At 24 h post transfection, most cells transfected with the PrV genome were detached from the culture well bottom compared with the control cells. The cell supernatant harvested was used to inoculate ST cells and the CPE gradually became obvious following continuous passages (data not shown). The indirect immunofluorescence indicated that there were green fluorescence signals on the surface of the infected cells. In contrast, no positive fluorescence signals were observed in mock-infected cells (Figure 1).

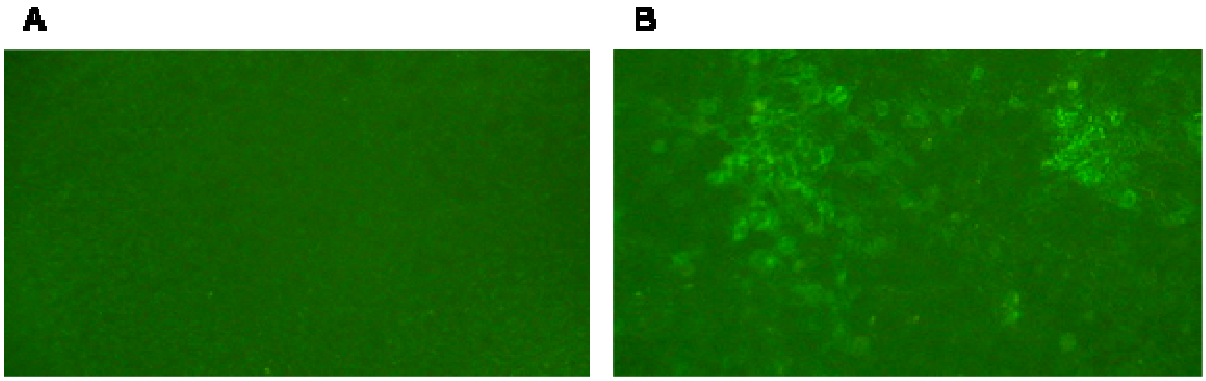

Figure 1. Immunofluorescence of swine testis (ST) cells infected with PrV strain Kaplan. Panel A is mock-infected ST cells, showing no specific fluorescence. Panel B shows the positive fluorescence of the PrV-infected ST cells.

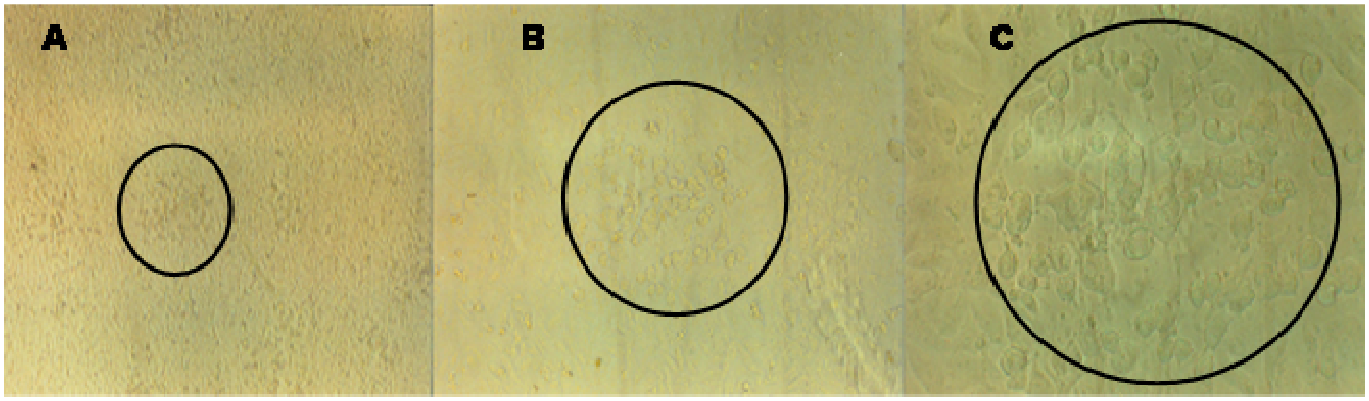

Figure 2. Plaque formation of Vero cells infected with PrV strain Kaplan. The circled regions in panels A to C are the same virus plaques under different magnitudes of enlargement of 40, 100 and 200, respectively.

CPE of PrV in Vero cells

To observe the CPE of PrV, the Vero cells were infected at an MOI of 10 with the virus. After absorption period, the medium was replaced with 1% methylcellulose. At 15 - 24 h.p.i, there were some significant CPE and the CPE numbers gradually increased after that. At 36 - 48 h.p.i., the CPE caused by PrV infection was easily visible and distinguishable. The typical CPE was characterized as that the infected cells detached from each other, became round and formed an approximate circular or oval plaque. In addition, PrV infection often resulted in the formation of large syncytia (Figure 2).

Comparison of growth kinetics between PrVs Kaplan and Bartha K-61

Both PrVs Kaplan and Bartha K-61 were used to infect Vero cells in 24-well plates. At 20 h.p.i., the cells infected by the two viruses displayed semblable CPE morphology and number (data not shown). The single-step growth kinetics indicated that both viruses had basically equal growth property (Figure 3), suggesting they may have a similar propagation ability and infectivity.

DISCUSSION

In this study, a calcium phosphate cell transfection technique was used to transfect the full-length genome of a DNA virus, PrV, into mammalian cells, resulting in the production of infectious viruses. It has been known for several decades that transfection of herpesvirus genomes is able to result in the production of infectious virus (Miller et al., 1979). Previously, using lipofectamin 2000™ (Invitrogen, USA), a cationic lipid-mediated transfection reagent, we had also gotten the production of infectious PrV by genome transfection into mammalian cells; however, the results of the current study indicated that calcium phosphate reagent was also effective for cell transfection. This method is much cheaper than lipid-mediated transfection technique. The cell density for PrV genome DNA transfection is important and high cell density may result in a low production of viral particles (data not shown). In our study, we found that 80%

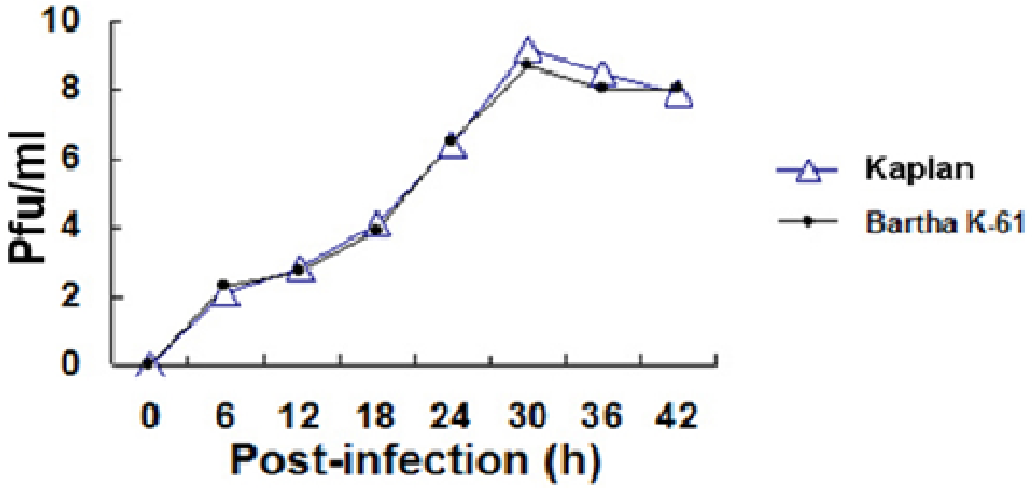

Figure 3. Single-step growth curves of PrVs Kaplan and Bartha K-61 on Vero cells. The cells were infected with the PrVs at a multiplicity of infection (MOI) of 10 and were incubated at 37℃. At 1 h.p.i. the cells were treated with low-pH citrate. At 6, 12, 18, 24, 30, 36 and 42 h.p.i, the viruses in the plates were harvested, and the virus titers expressed as plaque-forming unit (pfu)/ml were determined separately and plotted.

confluent cells transfected by PrV genome already produced PrV efficiently. To further identify the PrV, a polyclonal antibody derived from PrV Bartha K-61-immunized mice was used as primary antibody in the indirect immunofluorescence, and the results indicated that the rescued infectious virus shared similar immuno-reaction with PrV Bartha K-61.

The range of cell lines susceptible to PrV infection is very broad, and most of them such as PK15, ST, MARC-145, IBRS-2 and Vero cells can produce typical CPE (Whealy et al., 1992; Favoreel et al., 2002; Jusa et al., 1997; Yao et al., 2007; Yin et al., 2007). Among them, Vero cells, a well-characterized African green monkey kidney cell line, is suitable permissive cell for PrV infection. This cell line has been used for the production of various human virus vaccines, including those against poliomyelitis and rabies (Montagnon et al., 1987). By repeated addition of trypsin to the culture medium, it permits multicycle replication and the generation of high yields of influenza virus (Kaverin and Webster, 1995) and Vero cells can be used as an alternative host cell system for influenza A and B viruses (Govorkova et al., 1996). In addition, the propagation of porcine epidemic diarrhea virus (PEDV), a porcine coronavirus had been unsuccessful until serial passage of the virus in Vero cells (Hofmann and Wyler, 1988; Song et al., 2003). Therefore, this cell line is being widely used for virus research (Song et al., 2006; Ren et al., 2006; Lee et al., 2007). In this study, the CPE formed in PrV infected Vero cells was easily visible and distinguishable. Most infection produced some large syncytia, suggesting that the PrV infectivity was strong and CPE caused by PrV was very significant. Because the CPE formed in Vero cells infected by PrV is very typical, Vero cell-based plaque assays have been used in our previous report for quantitative analysis of PrV infection (Sui et al., 2010; Ren et al., 2010 b).

The PrV propagation property reflected by the single-step growth kinetics indicated that the PrV Kaplan shared similar growth property and plaque morphology with PrV vaccine strain Bartha K-61. Taken together, the results of this study clearly confirm that the transfection of PrV, a DNA viral genome into eukaryotic cells can produce the infectious PrV, providing the important experimental materials for relevant pathogenicity analysis of PrV. Although it has been reported that electroporation might be better than calcium phosphate precipitation as an efficient means of transfecting cultured cells with PrV genomic DNA (Luxton et al., 2005), the latter is more convenient and cheaper to produce infectious viruses. Especially, when PrV is requested for experimental purpose, it is better to transport the viral genome rather than live virus.

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