

# Replication Characteristics of Herpes Simplex Virus Type-1 (HSV-1) Recombinants in 3 Types of Tissue Cultures

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## ABSTRACT

A complication in the analysis of the role of ICP34.5 gene in the herpes simplex virus type-1 (HSV-1) lifecycle is the presence of overlapping antisense gene, open reading frame P (ORF P), which is also deleted in HSV-1 ICP34.5 negative mutants. A HSV-1 wild type strain (17<sup>+</sup>) ICP34.5/ORF P deletion mutant (1716) is totally avirulent in animal models and impaired in a number of *in vitro* functions: replication in 3T6 cells; and replication and shutoff of cellular protein synthesis in SK-N-SH cells. To attribute characteristics of 1716 to each of these two genes (ICP34.5 or ORF P), a number of HSV-1 recombinant viruses that express ICP34.5 and ORF P independently were constructed, purified and characterized. The parent of these recombinants is 1716 and they are (so called): 1622, expressing ICP34.5; 1624/24.5, expressing ORF P; and 1625, expressing both ICP34.5 and ORF P in separate loci. Using homologous recombination in cell culture, the recombinants were constructed and their DNA were analyzed by Southern-blotting. Expression of ICP34.5 and ORF P from the recombinants was checked by Western-blotting. Using cell culture, titration and plaque assay techniques, replication kinetics of the recombinants were compared with 17<sup>+</sup> and 1716 in 3 cell lines, BHK, 3T6, and SK-N-SH. The results showed that (i) ICP34.5 restored the ability to replicate and prevent host shutoff similar to wild type in SK-N-SH cells; (ii) ICP34.5 restored the replication phenotype to near wild type levels in 3T6 cells; and (iii) ORF P expression had no effect on the replication of these mutants. The characteristics of 1716 is obviously due to the lack of ICP34.5 and ORF P has no role in the characteristics studied. Thus, the function of ORF P still has to be determined. *Iran. Biomed. J.* 9 (3): 95-101, 2005

**Keywords:** Herpes simplex virus type-1 (HSV-1) recombinants, ICP34.5, Open reading frame P (ORF P)

## INTRODUCTION

Herpes simplex virus type-1 (HSV-1) is widely distributed in the human population and is the leading cause of acute sporadic viral encephalitis and viral induced blindness [1, 2]. HSV-1 is a neurotropic pathogen of humans that establishes a lifelong latent infection in the sensory ganglia innervating the site of primary infection. The capacity of the latent virus to reactivate is essential for its lifecycle and pathogenicity and a number of genes such as thymidine kinase are involved in virus pathogenicity and latency [3]. Several lines of evidence have mapped a neurovirulence locus to a gene, ICP34.5, in the long repeat region of the HSV-1 genome [4-8]. The

ICP34.5 gene encodes a protein consisting of 263 amino acids with a M<sub>r</sub> of 39 kDa in strain F [9, 10] and 248 amino acids with a M<sub>r</sub> of 37 kDa in strain 17<sup>+</sup> [11, 12]. ICP34.5 is essential *in vivo* [7]. ICP34.5 null mutants are unable to replicate in the central nervous system of mice and are totally avirulent with a LD<sub>50</sub> of 10<sup>6</sup> pfu/mouse higher than wild type or rescued virus [7, 8, 13]. In tissue culture, ICP34.5 null mutants replicate normally in many non-neuronal cell types such as Vero and BHK 21/C13 cells, but are restricted in SK-N-SH neuroblastoma and mouse embryo fibroblast (3T6) cells [7, 14-17]. In some non-permissive cells such as SK-N-SH, ICP34.5 null mutants induce a total shutoff of protein synthesis.

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In 1994, Lagunoff and Roizman [18] demonstrated that there are 16 open reading frames (ORF A-P) located in the opposite direction to ICP34.5 within the latency associated transcript (LAT) in the long repeat sequences in strain F. One of these, ORF P, map almost entirely antisense to ICP34.5. Detailed analyses of the products encoded by ORF P showed that ORF P expresses a 248-amino-acid protein in strain F and a 233-amino-acid protein in strain 17<sup>+</sup>. To date, no definite roles have been attributed to ORF P except a possible role in splicing at early times of infection [18, 19].

A HSV-1 17<sup>+</sup> spontaneous deletion mutant, 1716, which has a 759-bp deletion in the long repeat region, has been isolated and characterized [8]. In 1716, both copies of ICP34.5 and ORF P in the inverted repeat sequences are deleted. This mutant showed major behavioural differences both *in vivo* and *in vitro* compared to 17<sup>+</sup>. To assign the phenotypes of this mutant to either ICP34.5 or ORF P, a number of recombinant viruses based on 1716 were constructed. In these mutants, either only ICP34.5 or ORF P was inserted back into 1716 or both were inserted at separated loci and the phenotypes of these recombinants were analyzed. The recombinants are: 1622 expressing ICP34.5; 1624 and 1624.5 expressing ORF P in opposite orientation; and 1625 expressing both ORF P and ICP34.5 in separate loci. The goal of this work was to investigate the role of both ICP34.5 and ORF P in HSV-1 replication *in vitro*. An approach to achieve this idea is construction and *in vitro* characterization of a number of recombinants expressing these two genes.

## MATERIALS AND METHODS

**Cells.** Baby hamster kidney (BHK) cells were grown in Eagle's medium (Life technology, UK) supplemented with 10% (v/v) newborn calf serum [20]. Human neuroblastoma (SK-N-SH) cells and mouse embryo fibroblast 3T6 cells (European Tissue Culture Collection) were grown in Dulbecco's modified Eagle's medium (Life Technology, UK) supplemented with 10% (v/v) FCS.

**Viruses.** The wild type virus used was HSV-1 17<sup>+</sup> [21]. The HSV-1 17<sup>+</sup> deletion variant 1716 [8] was used as the parental virus for making recombinant viruses and as an ICP34.5 negative control in many experiments.

**Plasmids.** A number of plasmids used in this work were available in our Laboratory and are include: p35 ICP34.5, in which gD/ICP34.5 was (n.p. 94711) inserted into the HSV-1 UL43.5 and LacZ in U143 orientation in p35 [22, 23]. This plasmid was digested with *Xba*I to generate a linearized fragment containing gD ICP34.5/*LacZ* with flanking HSV DNA and was recombined with 1716 to generate 1622. p35/ORF P was constructed in the same manner as p35/ICP34.5. This plasmid was digested with *Xba*I to generate a linearized fragment containing gDORF P/*LacZ* with flanking HSV DNA and was recombined with 1716 to generate 1624.5. The same procedure was used to construct 1624. pAT5.1/gDORFP, which contains HSV-1 (n.p.136,289-139,258) US5 and gD/ORF P was cloned into the US5 plasmid. This plasmid was used to construct 1625. GST (Pharmacia, UK); GST-ORF P in which ORF P was inserted in frame at the end of GST.

**Generation of anti-ORF P sera.** ORF P was expressed as a fusion protein using the pGEX GST system [24]. The GST fusion protein was expressed in protease-deficient *E. coli* BL21 (Stratagene) and GST-ORF P was purified as previously described [24]. Two New Zealand white rabbits were injected with 1 mg of fusion protein in complete Freund's adjuvant, followed by three booster injections in incomplete Freund's adjuvant at 14-day intervals, and subsequently the blood samples were collected.

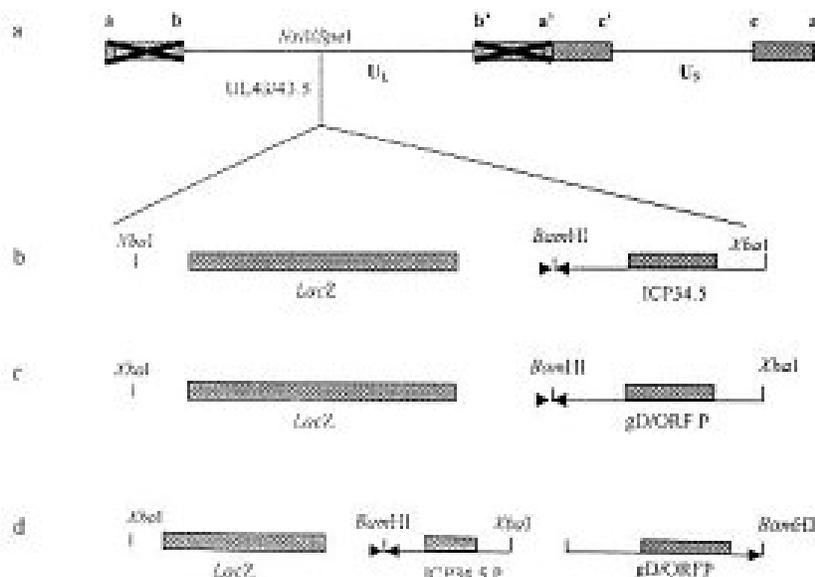
**Western-blotting.** Western-blotting was carried out essentially using anti-ICP34.5 [25] and anti-ORF P sera as described by McKay *et al.* [12].

**Southern-blotting.** Southern-blotting was carried out essentially as described by MacLean *et al.* [8].

**Multicycle replication kinetics.** Multicycle replication kinetics in BHK, 3T6 and SK-N-SH cells were determined using a m.o.i of 0.001 pfu/cell as described by MacLean *et al.* [8]. Samples were harvested at the designated time points (usually 0, 6, 12, 24, 48, 72 and 96 h) and titrated on BHK cells.

## RESULTS

**Construction of recombinant viruses.** All of the recombinant viruses used in this work have 1716 as their parent (Fig. 1a). The recombinant, 1622 expresses both gD/ICP34.5 and *LacZ* (Fig. 1b). To construct 1622, 1716 DNA was cotransfected with



**Fig. 1.** Structure of 1716, 1622, 1624.5 and 1625. **(a)**, This figure shows the structure of 1716. “a” is a direct repeat sequence present at both termini and in inverted orientation “a” in the internal repeat. “b” refers to the long and “c” to the short repeat. “X” indicates the deletion of ICP34.5/ORF P. The position of the *Nsi*I site plus additional *Spe*I site in UL43/43.5 is shown; **(b)**, This line shows the structure of 1622. ICP34.5/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.5 with ICP34.5 in the UL43.5 and *LacZ* in the UL43 orientation. The arrowheads indicate the orientation of transcription; **(c)**, This line shows the structure of 1624.5. gD/ORF P/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.5 with gD/ORF P in the UL43.5 and *LacZ* in the UL43 orientation. The arrowheads indicate the orientation of transcription. The 1624 also has the same structure; **(d)**, This line shows the structure of 1625. ICP34.5/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.5 and gD/ORF P was inserted into US5. The arrowheads indicate the orientation of transcription.

linearized p35/ICP34.5. The recombinant, 1624.5 expresses both gD/ORFP and *LacZ* (Fig. 1c). To construct 1624/1624.5, 1716 DNA was cotransfected with linearized p35/ORF P. 1625, whose parent is 1622, expresses both ICP34.5 and *LacZ*, and gD/ORF P being inserted into US5 (Fig. 1d). To construct 1625, 1622 DNA was cotransfected with linearized pAT5.1/ORF P. 1622 and 1624.5 were originally purified on the basis of their blue plaque morphology in the presence of X-gal and subsequently by their DNA profile, on Southern-blotting. The 1625, which has no additional colour marker, was isolated by its DNA profile, on Southern-blotting.

**Expression of ICP34.5 and ORF P from recombinant viruses.** By Western-blotting of BHK cell extracts with an anti-ICP34.5 serum it was demonstrated that 1622 and 1625 express ICP34.5. Also, by Western-blotting of BHK cell extracts with an anti-ORF P serum, it was shown that 1624, 1624.5 and 1625 express ORF P (data not shown).

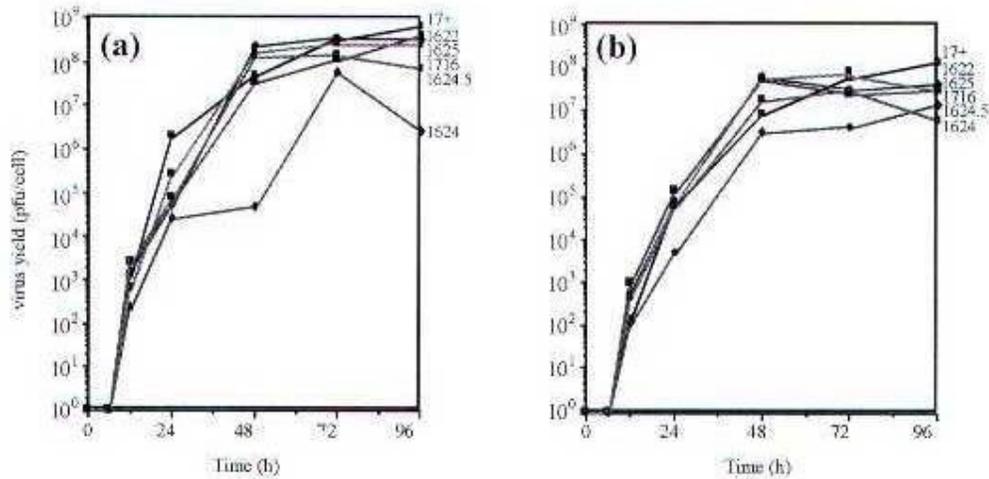
**Multicycle replication kinetics in BHK, 3T6 and SK-N-SH cells.** The aim of this part of work was to

determine the effect of inserting ICP34.5 or ORF P back into 1716.

In BHK cells, 17<sup>+</sup> and 1716 have been previously shown to grow identically [17]. Therefore as expected (Fig. 2), all viruses with the exception of 1624 exhibited a similar growth pattern, reaching a maximum titre of 10<sup>8</sup>-10<sup>9</sup> pfu/ml by 48-72 h pi.

In the stationary state of 3T6 cells, as is shown in Figure 3, 17<sup>+</sup> grew well reaching a maximum titre of 10<sup>6</sup>-10<sup>7</sup> pfu/ml by 48-72 h pi whereas 1716, expressing neither ICP34.5 nor ORF P failed to grow. The recombinants 1624 and 1624.5 which express only ORF P, similarly to 1716, failed to grow whereas 1622 and 1625 expressing ICP34.5 grew. Both 1622 and 1625 grew well and they reached a maximum titre of 10<sup>1</sup> to 10<sup>2</sup> fold lower than 17<sup>+</sup> (Fig. 2). However, in some experiments, both 1622 and 1625 grew similarly (Fig. 3a) whereas in others 1625 grew 10 fold less well than 1622. Most consistently, 1625 grew to a maximum titre 10 fold lower than 1622 (Fig. 3b).

In SK-N-SH cells (Fig. 4), 17<sup>+</sup> grew well reaching a maximum titre of 10<sup>7</sup>-10<sup>8</sup> pfu/ml by 48-72 h pi. The 1716 and 1624.5 reached a maximum titre



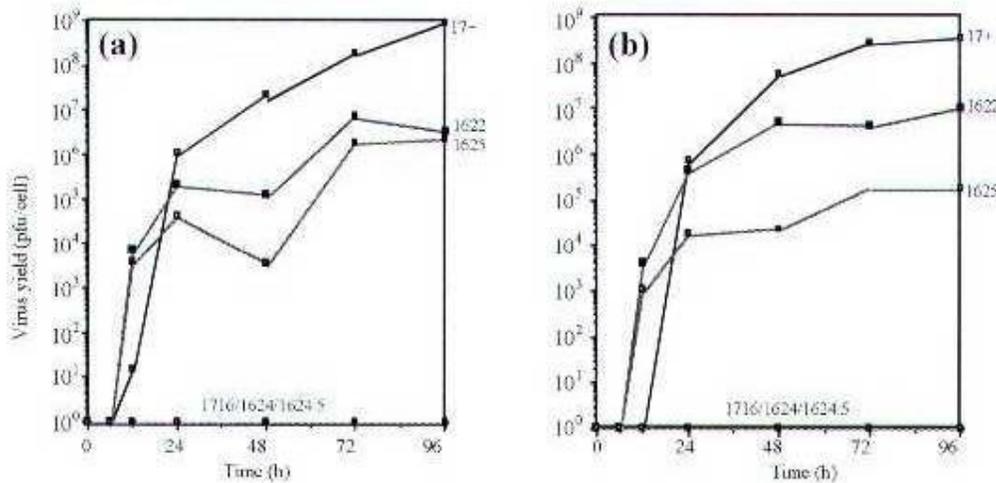
**Fig 2.** Multicycle replication kinetics in BHK cells. BHK cells were infected at a m.o.i of 0.001 pfu/cell. At 0, 6, 12, 24, 48, 72, and 96 h pi, infected cells were harvested. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. To obtain more reliable results, these experiments were carried out 2 times on each cell line. These separate experiments (a and b) are shown.

$10^4$  lower than  $17^+$ . In agreement with its impaired growth in BHK cells, 1624 grew consistently 10 fold poorer. Both 1622 and 1625 grew similarly to  $17^+$  (Fig. 4a). In some experiments, 1622 and 1625 grew similarly (Fig. 4a), whilst in others 1625 grew about 5 fold poorer than 1622 (Fig. 4 b).

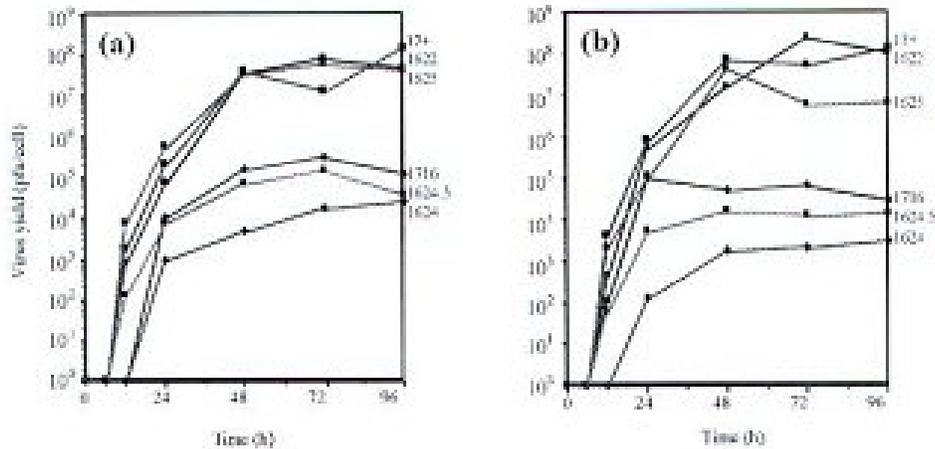
UL43/UL43.5 locus in the UL43.5 orientation. UL43/43.5 was selected as the site of insertion as both of them are non-essential genes [22]. In this cassette, the gene of interest was inserted under a HSV-1 gD promoter with a HSV-2 IE4/5 polyadenylation signal. In the opposite orientation, the *lacZ* gene was inserted under a SV40 promoter with its own polyadenylation signal. 1622 expresses gD/ICP34.5, 1624/1624.5 expresses gD/ORF P and 1625 expresses gD/ICP34.5 and also gD/ORF P in the non-essential US5 gene [4, 26].

### DISCUSSION

The basic design of all recombinant viruses was the insertion of an expression cassette into the 1716



**Fig. 3.** Multicycle replication kinetics in 3T6 cells. The 3T6 cells were infected at a m.o.i of 0.001 pfu/cell. At 0, 6, 12, 24, 48, 72, and 96 h pi infected cells were harvested. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. To obtain more reliable results, these experiments were carried out 2 times on each cell line. These separate experiments (a and b) are shown.



**Fig. 4.** Multi-cycle replication kinetics in SK-N-SH cells. SK-N-SH cells were infected at a m.o.i of 0.001 pfu/cell. At 0, 6, 12, 24, 48, 72, and 96 h pi infected cells were harvested. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. To obtain more reliable results, these experiments were carried out 2 times on each cell line. These separate experiments (a and b) are shown.

Western-blotting analysis of 1622 and 1625 infected BHK cells demonstrated the expression of ICP34.5 in levels compared with wild type 17<sup>+</sup>. 1624, 1624.5 and 1625 express low levels of ORF P protein. The antisera used were rabbit polyclonal sera which were raised against a GST-ORF P fusion protein. These antisera exhibited problems of low levels detection of ORF P from the recombinants and to a lesser extent from 17<sup>+</sup> but not from the overproducing *ts* mutant in ICP4 (*tsK*) at the non-permissive temperature (NPT).

It had been previously shown that neither ICP34.5 nor ORF P is essential for replication of HSV-1 17<sup>+</sup> in BHK cells [8]. Therefore, as expected in these cells, all the recombinant viruses, except 1624, had a similar growth pattern to wild type virus, confirming that the viruses had no secondary mutations effecting growth. The 1624 seems to be impaired in growth in BHK cells compared with the other viruses, indicating a probable secondary mutation elsewhere in the genome.

In stationary state, 3T6 cells, 1624 and 1624.5 behaved similarly to 1716 and failed to grow whereas 1622 and 1625 grew. In this cell line, replication of ICP34.5 null mutant (1716) has been previously shown to be severely impaired compared to 17<sup>+</sup> [16,17]. These workers also showed a similar result to this work, that in this non-permissive cells, the growth defect of ICP34.5 null mutants is greater when they are in a stationary phase [17]. One explanation is that non-permissive cells infected with ICP34.5 null mutants undergo a total shutoff of protein synthesis. Thus, the defect in 1716 was mainly overcome by the expression of ICP34.5 but

not ORF P. Expression of ORF P either by itself or in conjunction with ICP34.5 had no positive effect on 1716 growth.

The growth of 1716 in SK-N-SH cells had not been previously characterized. In SK-N-SH cells, 17<sup>+</sup> grew well reaching a maximum titre of 10<sup>7</sup>-10<sup>8</sup> pfu/10<sup>6</sup> cells. It has been suggested that the role of ICP34.5 is to maintain host and viral protein synthesis late in some cell lines such as SK-N-SH by blocking the shutoff of host protein synthesis [15]. As expected, based on the host protein synthesis shutoff phenotype, SK-N-SH cells are fully not permissive for 1716. However, our results showed that this cell line is semi permissive for 1716 exhibiting limited growth with a maximum titre of 10<sup>4</sup> fold lower than 17<sup>+</sup>. Two possibilities could explain this semi-permissive rather than non-permissive phenotype. As SK-N-SH cells are a heterogenous cell line and a small percentage of cells have dedifferentiated and reverted to fibroblasts, these dedifferentiated cells might fully support 1716 replication whilst the differentiated cells are non-permissive. Alternatively, host protein synthesis shutoff might not completely inhibit growth of 1716 in infected SK-N-SH cells allowing limited growth of 1716 in these cells. The 1624.5 grew similarly to 1716 and thus expression of ORF P has no positive effect on 1716 growth. In agreement with its impaired growth in BHK cells, 1624 grew consistently 10 fold poorer than 1716. Both 1622 and 1625 grew similarly to 17<sup>+</sup> and thus expression of ICP34.5 restores 1716 growth to 17<sup>+</sup> levels in SK-N-SH cells. It appears that the lack of growth of HSV-1 (17+) in non-permissive 3T6 and

SK-N-SH cells is due to the lack of ICP34.5. Obviously, ORF P has no role on growth of this strain on these cell lines. Therefore, the function of ORF P still has to be determined.

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