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Short Communication

Herpes simplex virus type 1 strain KOS carries a defective US9 and a mutated US8A gene

Alexandra Negatsch, Thomas C. Mettenleiter and Walter Fuchs

Institute of Molecular Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald–Insel Riems, Germany

Correspondence
Walter Fuchs
walter.fuchs@fli.bund.de

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The membrane protein encoded by the US9 gene of alphaherpesviruses plays an important role during virion assembly and transport in neurons. Here, we demonstrate that in herpes simplex virus type 1 (HSV-1) strain KOS, due to base substitutions, the predicted TATA-box of US9 is mutated, and a premature stop is present at codon 58 of US9, which contains 91 codons in other HSV-1 strains. The TATA-box mutation also removes the native stop codon of the adjacent US8A gene, leading to extension of the coding region from 160 to 191 codons. Northern blot analyses revealed reduced transcription of US9 in cells infected with HSV-1 KOS. Moreover, a US9-specific antiserum did not detect any gene products in Western blot and immunofluorescence analyses of KOS-infected cells, indicating that the truncated protein is not stable. In contrast, Western blot reactions of a pUS8A-specific antiserum confirmed enlargement of this protein in HSV-1 KOS.

The DNA genomes of members of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* contain long and short unique regions (U_L and U_S), which are separated by inverted repeat sequences (Pellett & Roizman, 2007). Whereas many of the genes encoded within the U_L regions are conserved throughout the entire virus family, the U_S regions exclusively contain alphaherpesvirus-specific genes lacking detectable homologues in other subfamilies. Moreover, the gene content of the U_S region varies considerably even between different alphaherpesviruses.

In the genomes of herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), herpes B virus, as well as canine, equine, feline and several avian alphaherpesviruses, the ORF US8, which encodes envelope glycoprotein gE, is partly overlapped or immediately followed by an additional protein-encoding ORF of unknown function, which has been previously designated US8A or US8.5 (Fig. 1a) (Davison, 2010; Georgopoulou *et al.*, 1993, 1995; McGeoch *et al.*, 1985; Perelygina *et al.*, 2003; Telford *et al.*, 1998; Tyack *et al.*, 1997; Willemse *et al.*, 1995). Also the adjacent ORF US9 of HSV-1 (Fig. 1a) is not conserved throughout the subfamily *Alphaherpesvirinae*, but absent e.g. from the lymphotropic avian pathogens of the genus *Mardivirus* (Davison, 2010; Tulman *et al.*, 2000).

All US8A and US9 homologues investigated to date are dispensable for virus replication in epithelial cell cultures. However, in the neuroinvasive mammalian alphaherpesviruses pseudorabies virus (PrV), bovine herpesviruses 1 and 5 (BoHV-1 and BoHV-5) and HSV-1, US9 has been reported to be critical for neuronal spread and neurovirulence (Brideau *et al.*, 2000; Butchi *et al.*, 2007; Chowdhury *et al.*, 2002; Polcicova *et al.*, 2005). Both US8A and US9 are predicted to encode type II membrane proteins (Davison, 2010; Sonnhammer *et al.*, 1998), and the US9 gene products (pUS9) of HSV-1, BoHV-5 and PrV are phosphorylated and incorporated into virions (Brideau *et al.*, 1998; Chowdhury *et al.*, 2002; Frame *et al.*, 1986). Detailed studies of PrV revealed that pUS9 localizes to the *trans*-Golgi network, and is specifically required for anterograde transport of nascent virus particles in neurons by targeting other viral envelope proteins, and, possibly indirectly, also nucleocapsids to axons (Brideau *et al.*, 2000; Lyman *et al.*, 2007; Tomishima & Enquist, 2001). Similar results have been described for the HSV-1 and BoHV-1 homologues (Butchi *et al.*, 2007; Snyder *et al.*, 2008). Conservation of pUS9 function was proven by complementation of US9-deleted PrV by the homologous proteins of equine herpesvirus 1 and BoHV-1, as well as of US9-deleted BoHV-5 by the BoHV-1 protein (Chowdhury *et al.*, 2006; Lyman *et al.*, 2009). However, pUS9 of HSV-1 or varicella-zoster virus (VZV) failed to compensate functionally for the PrV protein (Lyman *et al.*, 2009), and recent studies indicate that pUS9 is dispensable for anterograde axonal transport of HSV-1 particles, whereas the product of the US8 gene, gE, is required (McGraw *et al.*, 2009).

Since different results regarding the relevance of pUS9 for axonal transport of HSV-1 have been obtained using strains F (Snyder *et al.*, 2008) and NS (McGraw *et al.*, 2009), we attempted to investigate the role of this protein

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is HM188403.

Supplementary figures are available with the online version of this paper.

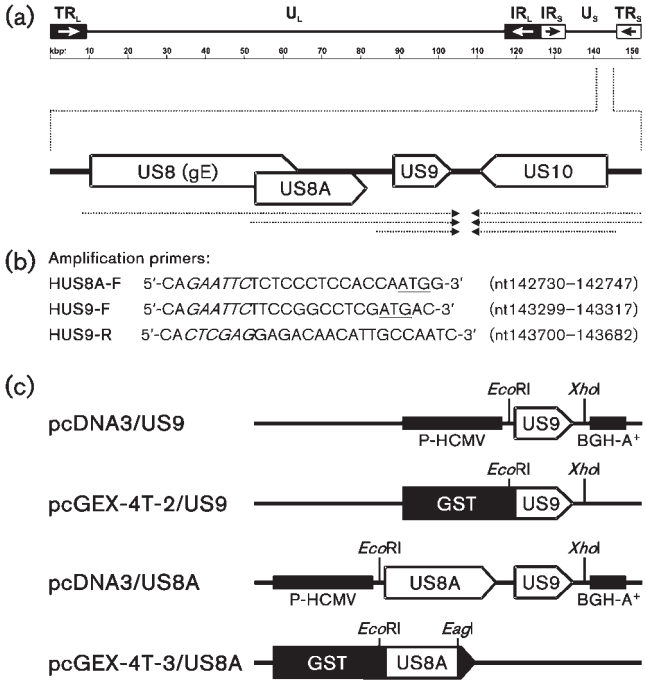

Fig. 1. Cloning of US8A and US9. (a) Schematic map of the HSV-1 genome containing long and short unique regions (U_L and U_S), bracketed by internal and terminal inverted repeats (TR_L, IR_L, IR_S and TR_S). An enlarged section of the U_S region shows ORFs (pointed rectangles) and viral transcripts (dotted arrows). (b) Primers used for amplification of US9 and US8A. The predicted start codons are underlined, and nucleotide positions in the HSV-1 genome (GenBank accession no. X14112) are given. The 5′-terminal extensions contain artificial *Eco*RI or *Xho*I restriction sites (italics). (c) These sites were used for insertion of the amplified ORFs into plasmid pcDNA3 (Invitrogen), which contains the human cytomegalovirus immediate-early promoter (P-HCMV) and the bovine growth hormone polyadenylation signal (BGH-A^+). US9 and a *Eco*RI–*Eag*I fragment containing codons 1–110 of US8A were also cloned into prokaryotic expression vectors pGEX-4T-2 or 3 (GE Healthcare) for expression of fusion proteins with glutathione *S*-transferase (GST).

for HSV-1 strains KOS (Smith, 1964) and 17 (Brown *et al.* 1973). The latter two HSV-1 strains were originally isolated from human labial lesions, and are frequently used as parental wild-type strains for the generation of virus mutants and investigation of viral gene functions (Brown *et al.*, 1973; Schaffer *et al.*, 1973). Moreover, bacterial artificial chromosomes (BAC) containing the infectious full-length genomes of both strains have been created to permit convenient mutagenesis in *Escherichia coli* (Gierasch *et al.*, 2006; Leege *et al.*, 2009), and strain 17 has been used for determination of the first complete genome sequence of HSV-1 (McGeoch *et al.*, 1985, 1988; GenBank accession no. X14112).

From this sequence, primers were deduced and used for PCR amplification (*Pfx* DNA Polymerase; Invitrogen) of the US8A and US9 genes of HSV-1 strains KOS and 17 (Fig. 1b). Using artificially introduced *Eco*RI and *Xho*I restriction sites, the PCR products obtained with primers HUS8A-F and HUS9-R, or HUS9-F and HUS9-R, were cloned into appropriately digested eukaryotic and prokaryotic expression vectors (Fig. 1c).

DNA sequence analyses of the obtained plasmid clones of HSV-1 strain 17 revealed 100 % identity with the published genome sequence (accession no. X14112). However, the newly determined DNA sequence of HSV-1 KOS (GenBank accession no. HM188403) exhibited three base substitutions within the investigated 971 bp fragment (Fig. 2a). Identical changes were found in two independently obtained isolates of HSV-1 KOS from different laboratories, as well as in two available BAC clones of this virus (Gierasch *et al.*, 2006; Leege *et al.*, 2009).

All three ‘mutations’ of HSV-1 strain KOS are predicted to affect viral gene expression. The first exchange at nucleotide position 142802 (numbering according to the HSV-1 strain 17 genome sequence) is a T to C transition, which leads to the substitution of a methionine by a threonine codon at US8A position 20. The second base substitution (nt 143221; T to C) abrogates the stop codon of US8A present in HSV-1 strain 17 (Fig. 2a). This leads to an extension of the deduced protein from 159 to 190 aa in strain KOS (Fig. 2b).

The latter mutation also affects the predicted TATA-box of US9 between nt 143219 and 143224 (McGeoch *et al.*, 1985), and reduces similarity to the consensus sequence $TATA^T/_AA^T/_A$ (Breathnach & Chambon, 1981). Furthermore, another base substitution (nt 143384; C to T) not only deletes a *Sal*I restriction site present in HSV-1 strain 17, but also introduces a stop codon at position 58 of US9 in HSV-1 strain KOS (Fig. 2a). This mutation shortens the deduced US9 protein from 90 aa in HSV-1 strain 17 to 57 aa in strain KOS, and prevents expression of the predicted hydrophobic C-terminal transmembrane domain (Fig. 2b). Thus, if expressed at all, pUS9 of HSV-1 strain KOS may not actually be functional as a membrane protein that localizes and targets other viral envelope proteins in neurons.

To test for differences in gene expression, RNA was prepared from cells infected with HSV-1 strain KOS or HSV-1 strain 17, and analysed by Northern blot hybridization (Fuchs & Mettenleiter, 1996) by using a labelled antisense cRNA of pcDNA3/US9 (Fig. 1c) specific for the 3′-co-terminal mRNAs of US8, US8A and US9 (Fig. 1a). This probe detected three viral transcripts of the expected sizes of approximately 2.5, 1.1 and 0.6 kb in cells harvested 6 h after infection with either virus (Fig. 3a). However, densitometric analyses revealed that the intensity of the US9-specific 0.6 kb transcript was reduced by approximately 65 % in HSV-1 strain KOS- compared with HSV-1 strain 17-infected cells, whereas the amounts of the two other transcripts were similar. This was also found at earlier and later times after infection (data not shown), indicating that the TATA-box mutation decreases US9 transcription.

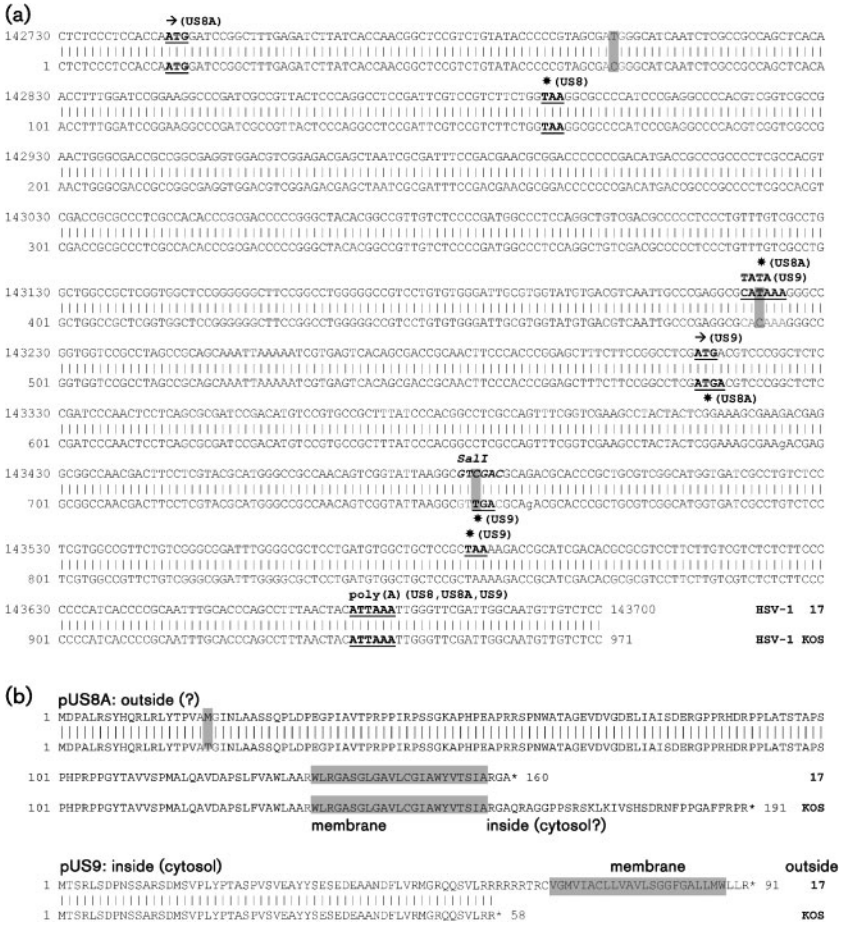

Fig. 2. Comparison of DNA (a) and deduced protein sequences (b) of HSV-1 strains 17 and KOS. Nucleotides are numbered according to the GenBank sequence (accession number X14112), and three base exchanges are highlighted. Putative transcriptional control signals (TATA-box and polyadenylation signal), start (→) and stop codons (✱) of US8, US8A and US9 are underlined, and a *SalI* restriction site specific for strain 17 is in italics. The predicted cytosolic, transmembrane (highlighted) and ectodomains of pUS8A and pUS9 are also indicated.

Fusion proteins containing pUS9 or the N-terminal 110 aa of pUS8A of HSV-1 strain 17 were isolated from bacteria that had been transformed with prokaryotic expression plasmids (Fig. 1c), and used for the preparation of monospecific rabbit antisera (Fuchs *et al.*, 2002). In Western blot analyses of cells infected with HSV-1 strain 17, the anti-US9 serum specifically detected several proteins with apparent molecular masses of 14–20 kDa, which were neither found in uninfected cells (Fig. 3b, upper panel), nor detected by the pre-immune serum (data not shown). Although the calculated mass of wild-type pUS9 is only 10.0 kDa, the observed masses are in agreement with previous findings (Frame *et al.*, 1986), and might be due to differential phosphorylation. Similar proteins were also detected in cells infected with several other HSV-1 strains (data not shown). However, no US9 gene products were detectable by Western blot in lysates of cells infected with HSV-1 strain KOS (Fig. 3b, upper panel), although a truncated US9 protein of the calculated mass of 6.4 kDa should be visible on the blot, and although sera against other viral gene products such as pUL11 (Leege *et al.*, 2009) showed similar reactivity with HSV-1 strain KOS- or 17-infected cells (Fig. 3b, lower panel). The pUS9-specific rabbit serum showed strong indirect immunofluorescence reactions with HSV-1 strain 17-infected cells, whereas only very weak signals were found in strain KOS-infected cells (Fig. 3c, left panels), which might have been caused by unspecific binding of antibodies to HSV-1 gE via their Fc-part (Para *et al.*, 1982).

Cells were also transfected with eukaryotic expression plasmids containing the US9 ORFs of HSV-1 strains KOS or 17 under human cytomegalovirus immediate-early promoter control (Fig. 1c). Again, only pUS9 of HSV-1 strain 17 was detectable in Western blot (Supplementary Fig. S1, available in JGV Online) and indirect immunofluorescence analyses (Supplementary Fig. S2, available in JGV Online), whereas no reaction was obtained with the US9 constructs of HSV-1 strain KOS despite similar transfection efficiencies (Supplementary Fig. S2).

To exclude the possibility that our polyclonal anti-US9 serum lacks antibodies against the N-terminal part of pUS9, which is preserved in the deduced gene product of HSV-1 strain KOS (Fig. 2b), cells were transfected with an expression plasmid encoding pUS9 aa 1–58 fused to EGFP. This fusion protein was stably expressed and detected by the anti-US9 serum in Western blot and immunofluorescence analyses (Supplementary Fig. S1). Thus, our findings indicate that stable expression of the mutated pUS9 of HSV-1 strain KOS is impaired. The protein might be either rapidly degraded or eliminated from the cells.

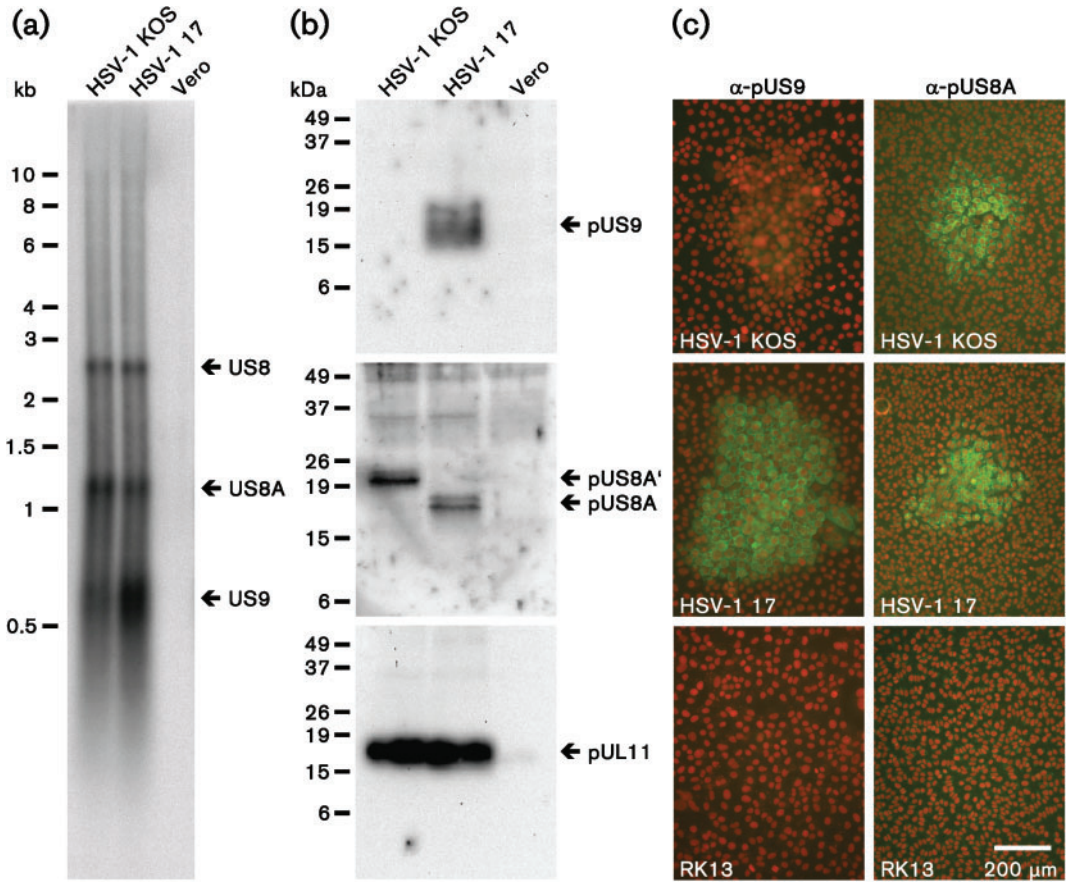

Fig. 3. Expression of the US8A and US9 genes of HSV-1 strains KOS and 17. (a) For Northern blotting, total RNA was prepared 6 h after infection of Vero cells at an m.o.i. of 10, and separated in a denaturing 0.8 % agarose gel. The blot was hybridized with a ^{32}P-labelled cRNA transcribed from pcDNA3/US9 with SP6 RNA polymerase, and analysed by radioluminography (FLA-3000; Fuji). Sizes of marker RNAs are indicated. (b) For Western blot analysis, Vero cells were harvested 20 h after infection at an m.o.i. of 5. Proteins were separated in discontinuous SDS-15 % polyacrylamide gels, and blots were incubated with monospecific rabbit antisera against pUS9 (upper panel, dilution 1 : 10 000), pUS8A (middle panel, dilution 1 : 10 000) or pUL11 (lower panel, dilution 1 : 100 000) of HSV-1. Reactions were detected by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Thermo Scientific) after incubation with peroxidase-conjugated secondary antibodies (Dianova). Molecular masses of marker proteins are indicated. (c) For indirect immunofluorescence analyses, rabbit kidney (RK13) cells were fixed with methanol and acetone (1 : 1) 48 h after infection at a low m.o.i. (<0.001). Binding reactions of the pUS9 or pUS8A-specific rabbit sera (dilutions 1 : 100) were detected after incubation with Alexa Fluor 488-conjugated secondary antibodies (green fluorescence; Invitrogen) and chromatin counterstaining with propidium iodide (red fluorescence).

Comparable amounts of the US8A proteins of HSV-1 strains 17 and KOS (pUS8A') were expressed in infected cells as shown by Western blot (Fig. 3b, middle panel), and immunofluorescence reactions (Fig. 3c, right panels) of the US8A-specific antiserum. In contrast to earlier data (Georgopoulou *et al.*, 1995), pUS8A was found in the cytoplasm and no nucleolar localization was observed. As expected, the sizes of the US8A proteins differed and were in agreement with the calculated molecular masses 20.2 or 16.8 kDa for strains KOS and 17, respectively. In both cases, at least two protein bands were detected (Fig. 3b, middle panel), which might be due to differential phosphorylation (Georgopoulou *et al.*, 1995).

In summary, all tested cell culture samples and BAC clones of HSV-1 strain KOS contain mutations affecting the US8A and US9 genes. Both genes also exhibit some variation in other HSV-1 strains. Compared to strain 17, the deduced US8A proteins of the clinical isolate H129 and of strain HF10 exhibit one and four amino acid substitutions, respectively (McGeoch *et al.*, 1985; Szpara *et al.*, 2010; Ushijima *et al.*, 2007). Likewise, the US9 proteins of HSV-1 strains 17 and HF10, and of isolate H129 differ at one or two positions.

However, two of the mutations in HSV-1 strain KOS are located at nucleotide positions crucial for the expression or

size and overall structure of the US8A and US9 gene products. A functional interdependence of these changes appears conceivable, since the two structurally related type II membrane proteins might contribute to related functions during virus replication. Like the preceding glycoprotein gene cluster (McGeoch, 1990), US8A and US9 may have evolved from a common ancestor, although no sequence homologies are detectable (Davison, 2010), Furthermore, computer predictions (e.g. TMpred; http://www.ch.embnet.org/software/TMPRED_form.html) suggest opposite membrane topologies for pUS8A and pUS9 of HSV-1 (Fig. 2b). Our antibodies might help to determine localization and orientation of pUS8A.

Although HSV-1 strain KOS is actually a phenotypic US9 null mutant, it replicates efficiently in cell culture and is neurovirulent in mice (Dix *et al.*, 1983). The described attenuation of certain KOS isolates has been mapped to genome regions other than the US9 gene (Thompson *et al.*, 1986). This might indicate a limited functional relevance of pUS9, which would be in line with the inconspicuous phenotypes of engineered US9 deletion mutants of HSV-1 and other alphaherpesviruses *in vitro* (Brideau *et al.*, 2000; Chowdhury *et al.*, 2002; Polcicova *et al.*, 2005), and partly also *in vivo* (Klopfleisch *et al.*, 2006; McGraw *et al.*, 2009; Nishiyama *et al.*, 1993).

Parallel investigations of different HSV-1 strains including KOS, targeted US9 and US8A mutants as well as rescuants in rodents and cultured neurons should finally elucidate the function of the US8 to US9 gene region in neurotropism and neurovirulence of alphaherpesviruses.

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