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Differences in the Regulatory and Functional Effects of the Us3 Protein Kinase Activities of Herpes Simplex Virus 1 and 2[▽]

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Us3 protein kinases encoded by herpes simplex virus 1 (HSV-1) and 2 (HSV-2) are serine/threonine protein kinases and play critical roles in viral replication and pathogenicity in vivo. In the present study, we investigated differences in the biological properties of HSV-1 and HSV-2 Us3 protein kinases and demonstrated that HSV-2 Us3 did not have some of the HSV-1 Us3 kinase functions, including control of nuclear egress of nucleocapsids, localization of UL31 and UL34, and cell surface expression of viral envelope glycoprotein B. In agreement with the observations that HSV-2 Us3 was less important for these functions, the effect of HSV-2 Us3 kinase activity on virulence in mice following intracerebral inoculation was much lower than that of HSV-1 Us3. Furthermore, we showed that alanine substitution in HSV-2 Us3 at a site (aspartic acid at position 147) corresponding to one that can be autophosphorylated in HSV-1 Us3 abolished HSV-2 Us3 kinase activity. Thus, the regulatory and functional effects of Us3 kinase activity are different between HSV-1 and HSV-2.

Us3 protein kinases encoded by herpes simplex virus 1 (HSV-1) and 2 (HSV-2) are serine/threonine protein kinases with amino acid sequences that are conserved in the subfamily *Alphaherpesvirinae* (6, 24, 36). Based on studies showing that recombinant Us3 mutants of HSV-1 and HSV-2 have significantly impaired viral replication and virulence in mice models, it has been concluded that both HSV-1 and HSV-2 Us3 protein kinases play important roles in viral replication and pathogenicity in vivo (25, 33, 41). In contrast, HSV-1 and HSV-2 Us3 protein kinases are not essential for growth in tissue culture cells (33, 36). Thus, recombinant Us3 mutants grow as well as wild-type viruses in Vero cells, and the mutants exhibit modestly impaired replication in HEp-2 cells (33, 36, 39, 40). The possible functions of Us3 have been extensively studied and gradually elucidated for HSV-1 Us3, but much less is known about HSV-2 Us3. These functions include (i) blocking apoptosis (1, 22, 30, 31, 35); (ii) promoting nuclear egress of progeny nucleocapsids through the nuclear membrane (39, 40, 45); (iii) redistributing and phosphorylating nuclear membrane-associated viral nuclear egress factors UL31 and UL34 (14, 37, 38) and cellular proteins, including lamin A/C and emerin (21, 27, 28); (iv) controlling infected cell morphology (13, 31, 32); and (v) downregulating cell surface expression of viral envelope glycoprotein B (gB) (12).

To determine the molecular mechanisms for a viral protein kinase's effects in infected cells, the kinase's physiological substrates and its phosphorylation sites must be identified. This can involve studies showing that the altered phenotypes observed in cells infected with a mutant virus lacking the protein kinase activity is also detected in cells infected with a mutant virus in which the substrate's phosphorylation sites have been modified by mutations. Although more than 15 potential HSV Us3 substrates have been reported, HSV-1 Us3 phosphorylation of only three substrates (Us3 itself, gB, and UL31) has been demonstrated to be linked directly with Us3 functions in infected cells (12, 13, 29, 41) as follows. (i) Us3 has been reported to autophosphorylate serine at position 147 (Ser-147), and this phosphorylation augments Us3's kinase activity in infected cells (13, 41). Even though only a small fraction of Us3 is autophosphorylated at Ser-147 in infected cells, alanine replacement of Ser-147 in Us3 significantly reduced HSV-1 replication in the mouse cornea and pathogenic manifestations of herpes stroma keratitis and periocular skin disease in mice (41). These results indicated that Us3 kinase activity was, in part, regulated by autophosphorylation of Ser-147, and regulation of Us3 activity by autophosphorylation played a critical role in viral replication in vivo and HSV-1 pathogenesis. (ii) It has been reported that HSV-1 Us3 phosphorylates Thr-887 in the cytoplasmic tail of gB, and this phosphorylation downregulates the cell surface expression of gB (12). Us3 phosphorylation of gB at Thr-887 also has been proposed to be involved in the regulation of fusion of the nascent progeny virion envelope with the cell's outer nuclear membrane, based on the observation that virions accumulated aberrantly in the perinuclear space in cells infected with mutant viruses carrying the amino acid substitution mutation T887A in gB and lacking the capacity to produce gH (45). The Us3 phosphorylation of gB at

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Thr-887 appeared to be critical for HSV-1 replication and pathogenesis in vivo, based on studies showing that the T887A substitution in the phosphorylation site in gB significantly reduced viral replication in the mouse cornea and pathogenic manifestations of herpes stroma keratitis and periocular skin disease in mice (Takahiko Imai, Ken Sagou, and Yasushi Kawaguchi, unpublished observations). (iii) It has been shown that Us3 phosphorylated some or all of the six serines in the UL31 N-terminal region, and this phosphorylation regulated the proper localization of UL31 and UL34 at the nuclear membrane and nuclear egress of nucleocapsids (29). Thus, the molecular basis of HSV-1 Us3 effects in infected cells have been gradually elucidated.

However, the Us3 phosphorylation sites in Us3 itself and in gB are not conserved between HSV serotypes (12, 13). The amino acid residues in HSV-2 Us3 and gB corresponding to HSV-1 Us3 Ser-147 and gB Thr-887 are aspartic acid (Asp-147) and alanine (Ala-887), respectively. These results suggest that some HSV-1 Us3 functions, such as regulation of its own catalytic activity and control of gB expression on the cell surface, are not regulated by HSV-2 Us3 or are regulated in a manner(s) different from HSV-1 Us3. In agreement with this suggestion, there is a marked difference between HSV-1 and HSV-2 virulence in mice following intracerebral infection, with the HSV-1 Us3 null mutant being $>10^4$-fold less virulent than the parent wild-type virus (25), while the HSV-2 Us3 null mutant was only ~10-fold less virulent (33). Although these results were from different reports and the mouse strains used in the studies were different, they indicate that some HSV-1 Us3 functions are different from those of HSV-2 Us3.

Therefore, we investigated differences in the biological properties of HSV-1 and HSV-2 Us3 protein kinases. It was of particular interest to examine whether Asp-147 in HSV-2 Us3 is required for its own kinase activity, since it is well established that acidic amino acids such as Asp or glutamic acid sometimes mimic the negative charges produced by phosphorylation (29, 46). In the present study, using a genetic manipulation system of HSV-2 with our newly constructed HSV-2 bacterial artificial chromosome (BAC) clone, we have shown that HSV-2 Us3 exhibited marked differences from HSV-1 Us3 in its catalytic functions, including the regulation of UL31/UL34 localization, nuclear egress of nucleocapsids, cell surface expression of gB, and virulence in mice. We also found that Asp-147 in HSV-2 Us3 was critical for its kinase activity, raising a possibility that the activity of Us3 kinases was regulated differently in HSV-1 and HSV-2.

MATERIALS AND METHODS

Cells and viruses. Vero and rabbit skin cells were described previously (44), as was HSV-1 wild-type strain HSV-1(F) (5, 44). HSV-2 wild-type strain 186 was described previously (1). Recombinant virus YK511 encoding an enzymatically inactive HSV-1 Us3 mutant, in which lysine at position 220 (Lys-220) was replaced with methionine (Us3K220M); recombinant virus YK513, in which a K220M mutation in YK511 was repaired; and recombinant virus YK304 which was reconstituted from pYEbac102, that contained a complete HSV-1(F) sequence with the BAC sequence inserted into the intergenic region between UL3 and UL4, were described previously (13, 44). YK304 has been shown to have a phenotype identical to that of wild-type HSV-1(F) in cell cultures and mouse models (44).

Construction of plasmids. A 4.0-kbp NotI-ClaI fragment of the HSV-2 186 viral genome, which contained genes UL50, UL51, and parts of UL49 and UL52, was cloned into pBluescript II KS(+) to yield pBS-2NC. To construct pBS246-GFP-BAC, a 1.6-kbp AseI-AflIII fragment of pEGFP-C1 (Clontech) containing an enhanced green fluorescent protein (EGFP) expression cassette (both sites were blunt ended) and a 6.4-kbp SalI fragment of pBelloBAC11 (Research Genetics) (the SalI sites were blunt ended) were cloned into the SmaI and EcoRV sites of pBS246 (Invitrogen), respectively. To generate pBS-2NC-EGFP/BAC, a NotI fragment of pBS246-GFP-BAC containing the BAC vector and EGFP expression cassette (the NotI sites were blunt ended) was cloned into a blunt-ended NdeI site in pBS-2NC.

Construction of recombinant virus YK351. To construct recombinant virus YK351 in which the BAC sequence and EGFP expression cassette, flanked by *loxP* sites, were inserted into the intergenic region between the HSV-2 UL50 and UL51 genes, rabbit skin cells were cotransfected with pBS-2NC-EGFP/BAC and HSV-2 186 viral DNA by using the calcium phosphate precipitation technique as described previously (43). At 3 or 4 days posttransfection, the transfected cells were harvested, freeze-thawed, and sonicated. The cell lysates were diluted and inoculated onto Vero cells, and plaques were screened for fluorescence with an inverted fluorescence microscope (Olympus IX71) (43). Recombinant viruses were plaque purified three times on Vero cells, and the virus genotypes were confirmed by Southern blotting.

Construction of an *Escherichia coli* strain harboring HSV-2-BAC. YK351 circular viral DNA was isolated from infected Vero cells by the Hirt method as described previously (44). To construct *E. coli* YEbac356 harboring YK351 circular viral DNA (pYEbac356), circular viral DNA from infected cells was electroporated into *E. coli* DH10B (Invitrogen), and transformed bacteria were grown on LB agar plates containing 12.5 μg of chloramphenicol/ml as described previously (44).

Mutagenesis of viral genomes in *E. coli* and reconstitution of recombinant HSV-2 from HSV-2-BAC. YK356 was generated by transfection of pYEbac356, isolated from YEbac356, into rabbit skin cells as described previously (44). To generate recombinant virus YK811, in which lysine at HSV-2 Us3 position 220 was replaced with methionine (Us3K220M), the two-step Red-mediated mutagenesis procedure was carried out using YEbac357, *E. coli* GS1783 (10) containing pYEbac356, as described previously (13), except with the primers 5′-TGATAGCAGCCACCCGAACTACCCTCATCGGGTAATCGTCATGGCGGGGTGGTACGCCAGAGGATGACGACGATAAGTAGGG-3′ and 5′-GCCGCGCCTCGTGGCTCGTGCTGGCGTACCACCCCGCCATGACGATTACCCGATGAGGGTCAACCAATTAACCAATTCTGATTAG-3′. As a result of the two-step Red-mediated mutagenesis procedure, *E. coli* strain YEbac811 containing the mutant HSV-2-BAC plasmid pYEbac811, with the K220M mutation in HSV-2 Us3, was obtained. YK811 was reconstituted from pYEbac811 as described above. To generate recombinant virus YK813 in which the K220M mutation in YK811 Us3 was repaired (Us3-KM-repair), the procedure to generate YK811 was used except with *E. coli* YEbac811 and primers 5′-TGATAGCAGCCACCCGAACTACCCTCATCGGGTAATCGTCAAGGCGGGGTGGTACGCCAGAGGATGACGACGATAAGTAGGG-3′ and 5′-GCCGCGCCTCGTGGCTCGTGCTGGCGTACCACCCCGCCTTGACGATTACCCGATGAGGGTCAACCAATTAACCAATCTGATTAG-3′. Recombinant virus YK815 carrying an alanine replacement of Asp-147 in HSV-2 Us3 (Us3-D147A) was constructed by the procedure used to generate YK811 except with primers 5′-TGTTCCGGGCCGCGCAGCCCCCCCCCGACGTCCGGACCTGCGCCAGCGGTAAGGTGGGGGCAGGATGACGACGATAAGTAGGG-3′ and 5′-CTTCCGGGGTGAACCCCGTGGCCCCCACCTTACCGCTGGCGCAGGTCCGGACGTCGGGGGCAACCAATTAACCAATTCTGATTAG-3′. To generate recombinant virus YK817 in which the D147A mutation in YK815 Us3 was repaired (Us3-DA-repair), the procedure to generate YK811 was used except with *E. coli* YEbac815, which was obtained in the procedure used to generate YK815 and contained pYEbac815 with a D147A mutation in Us3, and the primers 5′-TGTTCCGGGCCGCGCAGCCCCCCCCCGACGTCCGGACCTGCGACAGCGGTAAGGTGGGGGCAGGATGACGACGATAAGTAGGG-3′ and 5′-CTTCCGGGGTGAACCCCGTGGCCCCCACCTTACCGCTGTCGCAGGTCCGGACGTCGGGGGCAACCAATTAACCAATTCTGATTAG-3′.

BAC excision. To excise the BAC DNA (44), Vero cells were infected with recombinant adenovirus AxCANCre at a multiplicity of infection (MOI) of 100. At 24 h postinfection, cells were coinfected with YK356 at an MOI of 0.03, and the infection was allowed to proceed for another 24 h. Supernatants were then harvested, and YK361 was isolated from the supernatants and plaque purified once on Vero cells.

Animal studies. Three-week-old female ICR mice were purchased from Charles River. Mice were anesthetized with sodium pentobarbital and injected intracerebrally or intraperitoneally with 10-fold serial dilutions of each virus. Mice were monitored daily, and mortality from 1 to 21 days postinfection was attributed to the inoculated viruses. The 50% lethal dose (LD_{50}) values were calculated by the Behrens-Karber method.

Antibodies. Rabbit polyclonal antibody to Us3, VP22, UL11, UL34, UL31, and VP16 was described previously (13, 15, 20, 34). Mouse monoclonal antibody to gB (M27) was described previously (9). Mouse monoclonal antibody to gD (DL6) and ICP27 (8.F.137B) was purchased from Santa Cruz Biotechnology and Abcam, respectively. Rabbit polyclonal antibody to histone deacetylase 2 (HDAC2) was purchased from Sigma.

Southern blotting, immunoblotting, and immunofluorescence. Southern blotting, immunoblotting, and immunofluorescence were performed as described previously (11, 13, 17). The amount of protein in the bands in immunoblotting was quantitated by using the LAS-4000 with MultiGauge v3.11 software (Fuji Film).

Immune complex kinase assays. Immune complex kinase assays were performed as described previously (13), except that Vero cells infected with either HSV-2 186, YK811, YK813, YK815, or YK817 were subjected to immunoprecipitation. A fusion protein of maltose-binding protein (MBP) and BAD (MBP-BAD) expressed and purified as described previously (14) was used as a substrate. To reduce the possibility that the Us3 antibodies used in these studies might coprecipitate a protein kinase in addition to Us3, the precipitated material containing Us3 protein kinase was washed with a high-salt buffer containing 1 M NaCl prior to the assays, as described previously (13).

Detection of gB on infected cell surfaces by flow cytometry. Cell surface expression of gB in infected cells was detected by flow cytometry as described previously (12).

Electron microscopic analysis. Vero cells infected with HSV-2 186, YK811 or YK511 at an MOI of 5 for 18 h were fixed and processed for electron microscopic analysis as described previously (41).

RESULTS

Generation of a full-length infectious HSV-2 BAC clone. HSV-2 BAC clones that can be used in animal models have not been constructed thus far. Therefore, to study HSV-2 both in cell cultures and in vivo, we developed a genetic manipulation system of HSV-2 using a novel HSV-2-BAC clone that contains a full-length HSV-2 genome. We initially attempted to construct an HSV-2-BAC clone in which the BAC vector was inserted into the intergenic region between the HSV-2 UL3 and UL4 genes, because we previously had been able to construct a full-length infectious HSV-1 BAC clone by inserting the BAC vector into the intergenic region between HSV-1 genes UL3 and UL4 (44). However, it was difficult to construct a stable clone by inserting the BAC vector into the intergenic region between the HSV-2 UL3 and UL4 genes (data not shown). Therefore, we investigated inserting the BAC vector and EGFP expression cassette, flanked by *lox*P sites, into an insertion site in the intergenic region between the HSV-2 UL50 and UL51 genes, which we previously reported as a possible HSV site for insertion of foreign genes (26), and were able to construct a stable full-length HSV-2 BAC clone (pYEbac356) (Fig. 1).

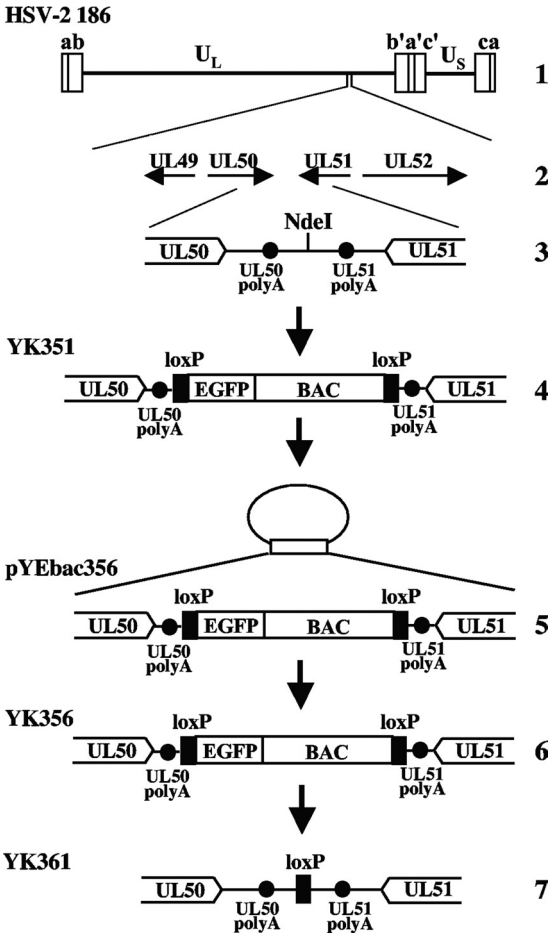

FIG. 1. Schematic diagram of genome structure of HSV-2 186 and the intergenic regions of recombinant viruses with or without the BAC sequence and the EGFP expression cassette insertions. Line 1, linear representation of the HSV-2 186 genome; line 2, structure of genome domain encoding genes UL49 to UL52; line 3, intergenic region between HSV-2 186 genes UL50 and UL51. The NdeI site used for insertion of the BAC sequence and EGFP expression cassette is shown. Lines 4, 6, and 7, schematic diagrams of recombinant viruses YK351, YK356, and YK361, respectively. Line 5, schematic diagram of plasmid pYEbac356.

Transfection of pYEbac356 into rabbit skin cells resulted in cytopathic effects (CPEs) and plaque formation, showing that pYEbac356 was infectious (data not shown). Viral DNAs from YK356, which was reconstituted from pYEbac356, and from wild-type HSV-2 186 were analyzed by RFLP with NotI. The pattern of NotI digestion of YK356 viral DNA was almost identical to that of wild-type HSV-2 186 viral DNA (Fig. 2A), with the difference being that, compared to NotI digested HSV-2 186 DNA, NotI-digested YK356 viral DNA had a 12.7-kb fragment (fragment a in Fig. 2A) and was missing a 4.3-kb fragment (fragment b in Fig. 2A) due to the BAC insertion.

To investigate the similarities between YK356 (HSV-2-BAC) and wild-type HSV-2 186 further, the expression of several YK356 (HSV-2-BAC) viral proteins was compared to that of HSV-2 186 by infecting Vero cells with YK356 (HSV-2-BAC) or HSV-2 186 at an MOI of 3 for 16 h and then assaying infected cells for the appropriate proteins by immunoblotting. The viral proteins examined were expressed at a similar level in YK356 and wild-type HSV-2 186 (Fig. 2B).

Since HSV-1 Ori sequences have been reported to be unstable in bacteria (2), we examined the stability of HSV-2 OriL and OriS sequences. For the present study, a fragment containing OriL and OriS was amplified by PCR from the HSV-2 186 and YK356 (HSV-2-BAC) genomes. The electrophoretic mobilities of the PCR products containing OriL and OriS amplified from YK356 (HSV-2-BAC) were identical to those amplified from wild-type HSV-2 186 (Fig. 2C), indicating that these origin sequences were stable in YEbac356. The conclusion from these experiments was that a full-length infectious HSV-2 186 genome had been cloned as a BAC plasmid.

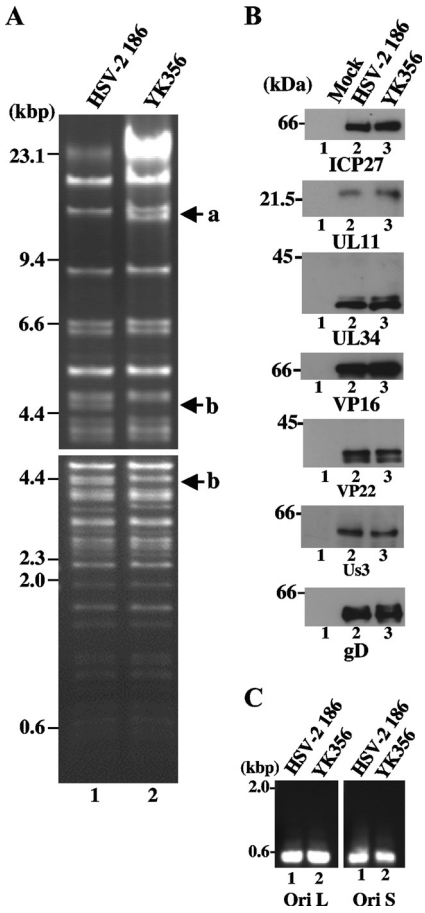

FIG. 2. (A) Agarose gel electrophoresis of NotI-digested viral DNAs of HSV-2 186 (lane 1) and recombinant virus YK356 (lane 2). Fragment a (~12.7 kb) and fragment b (~4.3 kb) were detected in the YK356 and HSV-2 186 genomes, respectively, as a result of insertion of the BAC sequence and EGFP expression cassette. (B) Immunoblots of electrophoretically separated lysates from Vero cells mock infected (lane 1) or infected with HSV-2 186 (lane 2) or YK356 (lane 3) at an MOI of 3. Infected cells were harvested at 16 h postinfection and immunoblotted with antibody to ICP27, UL11, UL34, VP16, VP22, Us3, or gD as indicated under each panel. (C) Agarose gel electrophoresis of PCR products containing OriL (left panel) and OriS (right panel) of HSV-2 186 (lane 1) and YK356 (lane 2). Molecular sizes are indicated on the left of panels A, B, and C.

The *lox*P sites were used to make it possible to remove the BAC and EGFP expression cassette sequences by Cre/Lox site-specific recombination (44). To examine whether the BAC vector and EGFP expression cassette flanked by *lox*P sites could be excised from the viral genome by Cre recombinase, Vero cells were infected with recombinant adenovirus AxCANCre (44) expressing Cre recombinase and then coinfected with YK356 (HSV-2-BAC). At 24 h postinfection, the supernatant was harvested and plaque purified once on Vero cells to yield YK361 (HSV-2-ΔBAC) (Fig. 1, line 7). Excision of the BAC sequence and EGFP expression cassette from the viral genome was confirmed by Southern blotting (data not shown) and fluorescence microscopy (data not shown). Furthermore, sequence analysis of the intergenic region between the YK361 (HSV-2-ΔBAC) UL50 and UL51 genes showed that the expected Cre-mediated excision had occurred and only one *lox*P site remained in the region (data not shown). These results indicated that the BAC vector and EGFP expression cassette sequences can be easily removed from the viral genome in Vero cells by coinfection with AxCANCre-expressing Cre recombinase.

Characterization of recombinant viruses reconstituted from pYEbac356. Viruses YK356 (HSV-2-BAC) and YK361 (HSV-2-ΔBAC) reconstituted from the full-length infectious HSV-2 186 BAC plasmid (pYEbac356) were characterized by two series of experiments. In the first series of experiments, Vero cells were infected with YK356 (HSV-2-BAC), YK361 (HSV-2-ΔBAC), or wild-type HSV-2 186 at an MOI of 3 or 0.01, and secreted extracellular virus and cell-associated virus were harvested separately as a function of time. The titer of each sample was then determined by a standard plaque assay on Vero cells. The growth curves of extracellular and cell-associated YK356 (HSV-2-BAC) and YK361 (HSV-2-ΔBAC) viruses were almost identical to those of wild-type HSV-2 186 (Fig. 3). In the second series of experiments, mice were injected intracerebrally or intraperitoneally with 10-fold dilutions of HSV-2 186, YK356 (HSV-2-BAC), or YK361 (HSV-2-ΔBAC), and mortality was monitored for 21 days postinfection. The LD_{50} values of YK356 and YK361 for both intracerebral and intraperitoneal infection were similar to those for wild-type HSV-2 186 (Table 1). These results indicated that, for the properties examined in these studies, YK356 (HSV-2-BAC) and YK361 (HSV-2-ΔBAC) viruses reconstituted from pYEbac356 retained wild-type growth kinetics in cell culture and virulence in mice.

Effect of alanine substitution for Asp-147 in HSV-2 Us3 on kinase activity. To investigate the role of HSV-2 Us3 Asp-147 on its protein kinase activity in infected cells, we generated recombinant viruses YK815, in which HSV-2 Us3 Asp-147 was replaced with alanine, and YK817, in which the D147A mutation was repaired (Fig. 4). We also generated recombinant viruses YK811 carrying a methionine substitution for HSV-2 Us3 Lys-220 and YK813 in which the K220M mutation was repaired (Fig. 4). HSV-2 Us3 Lys-220 corresponds to an invariant lysine in the known serine/threonine kinases and is in the active site involved in positioning the ATP γ-phosphate to facilitate phosphotransfer (7). Therefore, an amino acid substitution of the invariant lysine was expected to produce loss of Us3 protein kinase activity. In agreement with this expectation, substitution mutations in the corresponding lysines in HSV-1 Us3 and UL13, human cytomegalovirus UL97, and Epstein-Barr virus BGLF4 resulted in the loss of protein kinase activity (8, 14, 16). The genotypes of YK811 (Us3K220M), YK813

TABLE 1. LD_{50} values of wild-type HSV-2, YK356, and YK361

Virus	LD_{50} (PFU)[a]	
	Intracerebral	Intraperitoneal
HSV-2 186	2.2	146
YK356	3.2	68
YK361	3.2	100

[a] Three-week-old female ICR mice were inoculated intracerebrally or intraperitoneally, as indicated, with serial 10-fold dilutions of each virus in groups of six per dilution and monitored for 21 days. The LD_{50} values were determined by the Behrens-Karber method.

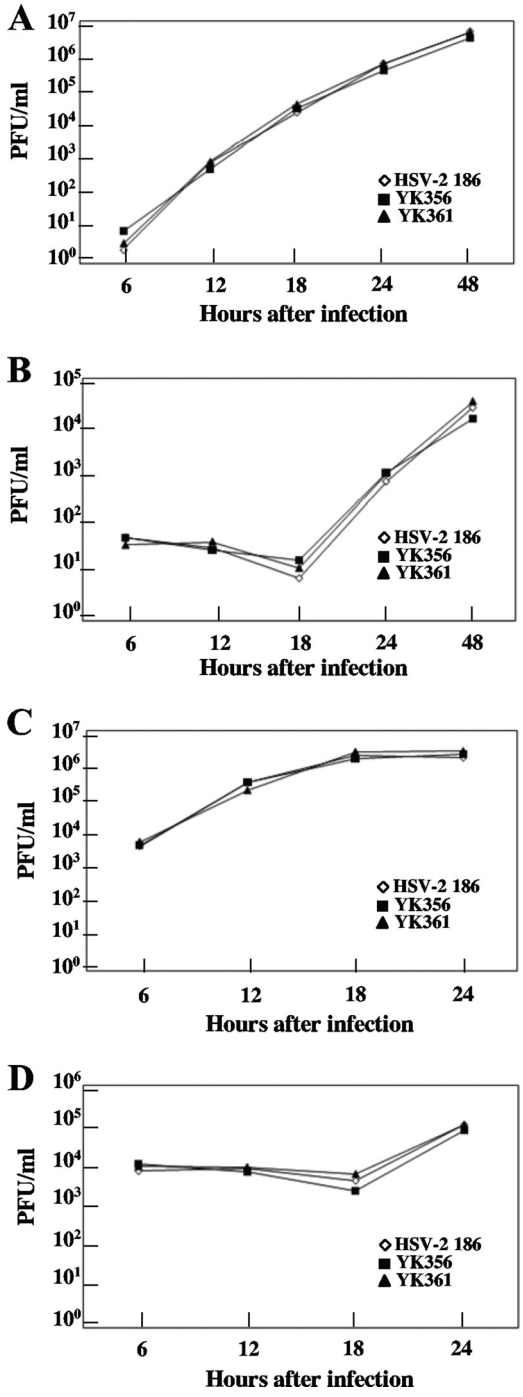

FIG. 3. Growth curves for recombinant viruses with or without the BAC sequence and EGFP expression cassette in their genomes. Vero cells were infected with HSV-2 186, YK356, or YK361 at an MOI of 0.01 (A and B) or 3 (C and D). Cell-associated virus from infected cells (A and C) and extracellular virus from cell supernatants (B and D) were harvested at the indicated times and assayed on Vero cells.

(Us3-KM-repair), YK815 (Us3D147A), and YK817 (Us3-DA-repair) were confirmed by PCR analyses and sequencing (data not shown). Expression of the predicted Us3 proteins was confirmed by infecting Vero cells with wild-type virus or one of the recombinant viruses at an MOI of 3 for 18 h and then assaying the infected cells for the appropriate proteins by immunoblotting with anti-Us3 antibody (Fig. 5A). YK811 (Us3K220M) and YK815 (Us3D147A) exhibited growth properties similar to those of wild-type HSV-2 186 at MOIs of 3 (Fig. 5B) and 0.01 (Fig. 5C) on Vero cells.

To examine the protein kinase activity of Us3 carrying a K220M or D147A mutation in infected cells, Vero cells infected with wild-type HSV-2 186, YK811 (Us3K220M), YK813 (Us3-KM-repair), YK815 (Us3D147A), or YK817 (Us3-DA-repair) at an MOI of 3 were harvested at 18 h postinfection, solubilized, and immunoprecipitated with anti-Us3 antibody. The immunoprecipitates were then used in in vitro kinase assays with purified MBP-BAD as a substrate. BAD is a pro-apoptotic cellular protein and has been reported to be a nice substrate for HSV-1 Us3 in in vitro kinase assays (14). As shown in Fig. 6, MBP-BAD was phosphorylated in kinase assays by Us3 immunoprecipitates from wild-type HSV-2 186-infected cells. Consistent with the effect of the K220M mutation in HSV-1 Us3 previously reported (13, 14), MBP-BAD was not phosphorylated by HSV-2 Us3K220M protein immunoprecipitated from YK811 (Us3K220M)-infected cells. However, MBP-BAD was phosphorylated by Us3 immunoprecipitates from cells infected with YK813, in which the K220M mutation in Us3 was repaired. These results indicated that HSV-2 Us3K220M was a kinase-negative mutant. Similarly, MBP-BAD was not phosphorylated by HSV-2 Us3D147A immunoprecipitated from YK815 (Us3D147A)-infected cells. However, MBP-BAD was phosphorylated by Us3 immunoprecipitates from cells infected with YK817, in which the D147A mutation in Us3 was repaired. These results indicated that Asp-147 in HSV-2 Us3 was essential for its kinase activity in infected cells.

It has been reported that HSV-1 Us3 catalytic activity is required for induction of posttranslational modification of HDAC2 and for that of wild-type HSV-1 CPEs at 24 h postinfection (13). Therefore, to investigate the role of HSV-2 Us3 kinase activity in these processes, Vero cells were infected with wild-type HSV-2 186, YK811 (Us3K220M), YK813 (Us3-KM-repair), YK815 (Us3D147A), or YK817 (Us3-DA-repair) at an MOI of 3, harvested at 18 h postinfection, and analyzed by immunoblotting with anti-HDAC2 antibody. In addition, CPEs of Vero cells infected with each of the viruses for 24 h were observed. As shown in Fig. 7A, in mock-infected Vero cells, HDAC2 appeared to be detected as two bands in a denaturing gel. The fast-migrating band was the dominant one, while a slower-migrating band was detected only faintly. As reported earlier for HSV-1 Us3, infection of Vero cells with wild-type HSV-2 186 or YK813 (Us3-KM-repair) caused significant increase in the amount of protein in the more slow-migrating band (Fig. 7). In contrast, in cells infected with recombinant HSV-2 expressing enzymatically inactive Us3 (YK811), the increase in the abundance of HDAC2 protein in the slow-migrating band was not observed, similar to what has been observed in mock-infected cells (Fig. 7). Furthermore, infection of Vero cells with wild-type HSV-2 186 or YK813 (Us3-KM-repair) efficiently induced cell rounding, while YK811 (Us3K220M) infection did so only partially (Fig. 8). These results indicated that, like HSV-1 Us3, HSV-2 Us3 kinase activity was required for modification of HDAC2 and induction of wild-type CPEs at 24 h postinfection. The phenotype of

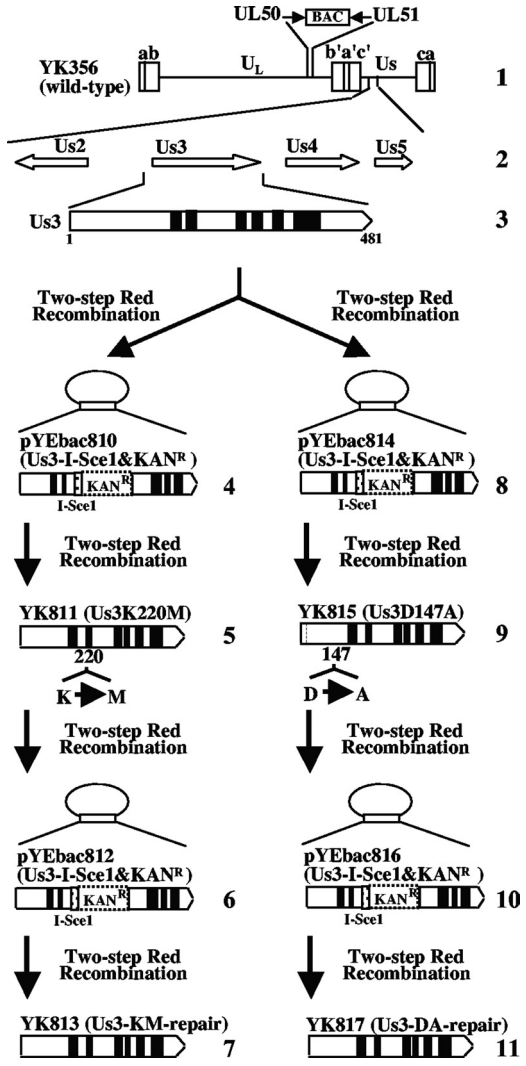

FIG. 4. Schematic diagram of the genome structure of YK356 and relevant domains of the recombinant viruses derived from it. Line 1, linear representation of the YK356 genome; line 2, structure of the genome domain containing the Us2, Us3, Us4, and Us5 open reading frames; line 3, putative amino acid sequence of Us3. The shaded areas represent subdomains I to VI, which are conserved in eukaryotic protein kinases (42). Lines 4, 6, 8, and 10, schematic diagram of plasmids pYEbac810, pYEbac812, pYEbac814, and pYEbac816; lines 5, 7, 9, and 11, schematic diagrams of recombinant viruses YK811, YK813, YK815, and YK817, respectively.

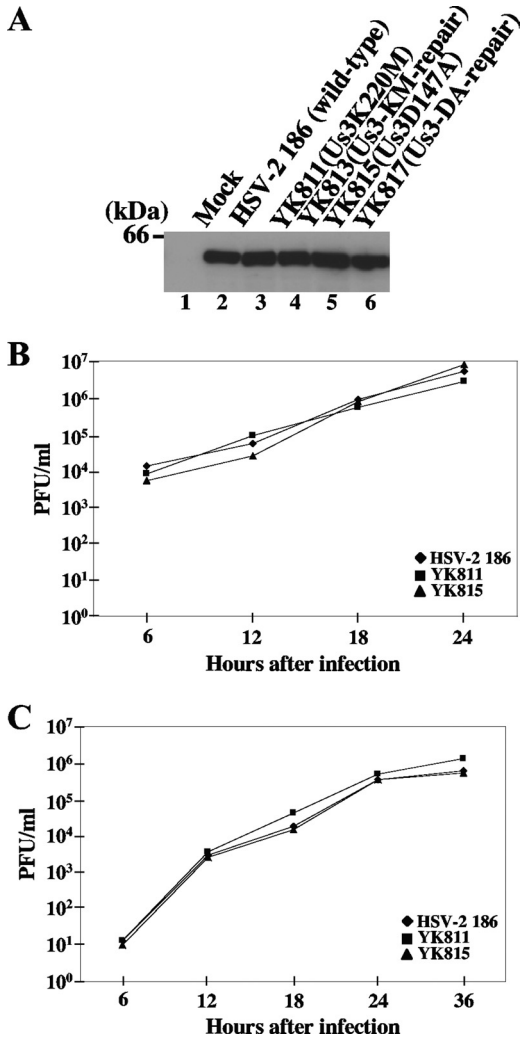

FIG. 5. (A) Immunoblot of electrophoretically separated lysates of Vero cells mock-infected (lane 1) or infected with HSV-2 186 (wild-type) (lane 2), YK811 (Us3K220M) (lane 3), YK813 (Us3-KM-repair) (lane 4), YK815 (Us3D147A) (lane 5), or YK817 (Us3-DA-repair) (lane 6) at an MOI of 3. The infected Vero cells were harvested at 18 h postinfection and immunoblotted with antibody to Us3. (B and C) Growth curves of wild-type and recombinant viruses. Vero cells were infected with wild-type HSV-2 186 (◆), YK811 (Us3K220M) (■) or YK815 (Us3D147A) (▲) at an MOI of 3 (B) or 0.01 (C). For each experiment, total virus from infected cells and cell culture supernatants was harvested at the indicated times and assayed on Vero cells.

the HSV-2 recombinant virus carrying a D147A mutation in Us3 (YK815) with respect to HDAC2 modification and CPE induction in infected cells was identical to that of YK811 (Us3K220M), and the wild-type phenotype was restored in Vero cells infected with YK817 in which the D147A mutation in HSV-2 Us3 was repaired (Fig. 7 and 8). Taken together, these results support our conclusion that Asp-147 in HSV-2 Us3 is essential for its kinase activity in infected cells.

Role of HSV-2 Us3 kinase activity in the regulation of localization of UL31 and UL34, nuclear egress of nucleocapsids, and cell surface expression of gB. To compare the biological effects of HSV-1 and HSV-2 Us3 kinase activities, three series of experiments were performed. In the first series of experiments, Vero cells were infected with wild-type HSV-1 YK304 (HSV-1-BAC), HSV-1 YK511 (Us3K220M), wild-type HSV-2 186, wild-type HSV-2 YK356 (HSV-2-BAC), or HSV-2 YK811 (Us3K220M) at an MOI of 3 for 12 h and localization of UL31 and UL34 proteins in infected cells was analyzed by immunofluorescence with confocal microscopy. HSV-1 UL34 and UL31 proteins are nuclear egress factors (38, 39), physiological substrates of Us3 (14, 37), and require HSV-1 Us3 kinase activity for proper localization at the nuclear envelope (13, 38, 40). As shown in Fig. 9, UL34 and UL31 proteins localized mainly at the nuclear envelope with a uniform distribution in Vero cells infected with wild-type HSV-1 (YK304) but were incorrectly localized as punctate structures in cells infected

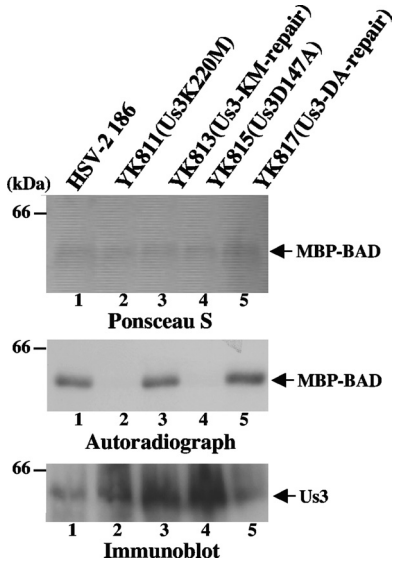

FIG. 6. In vitro kinase assays with Us3 immunoprecipitates from cells infected with wild-type and recombinant viruses. Vero cells were infected with wild-type HSV-2 186 (lane 1), YK811 (Us3K220M) (lane 2), YK813 (Us3-KM-repair) (lane 3), YK815 (Us3D147A) (lane 4), or YK817 (Us3DA-repair) (lane 5) at an MOI of 3; harvested at 18 h postinfection; and solubilized. Part of each lysate was analyzed by immunoblotting with anti-Us3 antibody (lower panel). The remainder of each lysate was immunoprecipitated with anti-Us3 antibody. The immunoprecipitates were incubated in kinase buffer containing [γ-^{32}P]ATP and MBP-BAD, separated in a denaturing gel, transferred to a nitrocellulose membrane, stained with Ponceau S (upper panel), and analyzed by autoradiography (middle panel).

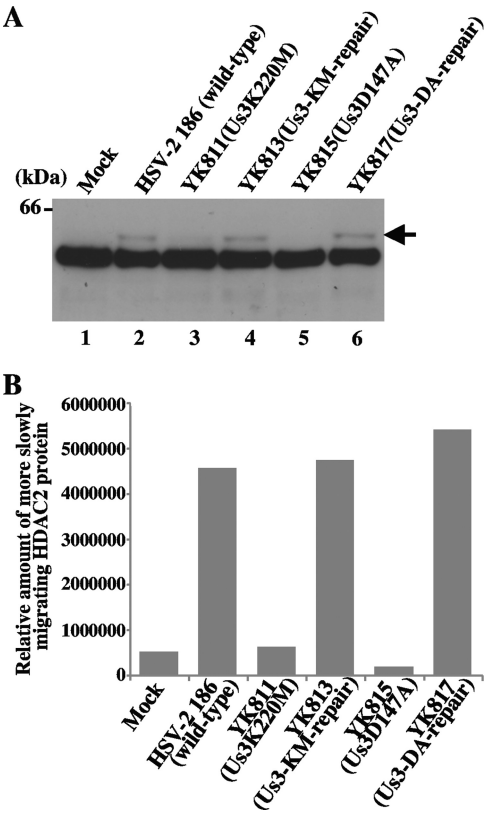

FIG. 7. (A) Immunoblot of lysates from Vero cells mock-infected (lane 1) or infected with wild-type HSV-2 186 (lane 2), YK811 (Us3K220M) (lane 3), YK813 (Us3-KM-repair) (lane 4), YK815 (Us3D147A) (lane 5), or YK817 (Us3-DA-repair) (lane 6) at an MOI of 3. Infected cells were harvested at 18 h postinfection, lysed, electrophoretically separated, and immunoblotted with anti-HDAC2 antibody. Posttranslationally modified HDAC2 is indicated by the arrow. (B) Quantitation of the amount of posttranslationally modified HDAC2 proteins detected with anti-HDAC2 antibody shown in panel A.

with YK511 (Us3K220M), a kinase-inactive HSV-1 Us3 mutant, in agreement with previous reports (13, 40). In Vero cells infected with wild-type HSV-2 186 and YK356, UL34 and UL31 proteins localized at the nuclear envelope in a uniform distribution as in wild-type HSV-1-infected cells (Fig. 9). In contrast to the observation with HSV-1 Us3 kinase-inactive viruses (13, 40), HSV-2 UL34 and UL31 proteins in Vero cells infected with YK811, a kinase-inactive HSV-2 Us3 mutant, showed the same localization pattern as in cells infected with wild-type HSV-2 186 and YK356 (Fig. 9).

In the second series of experiments, Vero cells were infected with wild-type HSV-2 186, HSV-2 YK811 (Us3K220M), or HSV-1 YK511 (Us3K220M) at an MOI of 5 for 18 h and then fixed and processed for electron microscopy. It has been reported that, in the absence of HSV-1 Us3 kinase activity, enveloped virions accumulate within membranous structures which appear to be invaginations of the inner nuclear membrane into the nucleus, probably leading to a delay in transit from the nucleus to the cytoplasm, while membranous invaginations were barely observed in wild-type HSV-1-infected cells (40). In agreement with that report, membranous invaginations retaining several enveloped virions were frequently observed in the nucleus of Vero cells infected with the HSV-1 YK511 recombinant virus encoding a Us3 kinase-inactive mutant (Fig. 10). However, membranous invaginations were observed much more rarely in Vero cells infected with wild-type HSV-2 186 and HSV-2 YK811 (Us3K220M) (Fig. 10). When 20 cells infected with each of the viruses described above were examined and membranous invaginations and enveloped virions retaining the structures were counted, most (95%) of HSV-1 YK511 (Us3K220M)-infected cells induced total 94 membranous invaginations, and 521 enveloped virions were detected in the structures. In contrast, none of cells infected with wild-type HSV-2 induced membranous invaginations. Similarly, in HSV-2 YK811 (Us3K220M)-infected cells examined, most (90%) of infected cells did not induced membranous invaginations. The remaining 10% of HSV-2 YK811 (Us3K220M)-infected cells induced in all only three membranous invaginations, and 15 enveloped virions were detected in the structures.

In the third series of experiments, Vero cells were infected with wild-type HSV-1(F), HSV-1 YK511 (Us3K220M), wild-type HSV-2 186, or HSV-2 YK811 (Us3K220M) at an MOI of 3 and harvested at 6 h postinfection. Expression of gB on the cell surface of infected cells was analyzed by flow cytometry. In agreement with a previous report (12), cell surface expression of gB in Vero cells infected with HSV-1 YK511 (Us3K220M) was upregulated compared to that in wild-type HSV-1 (F)-infected cells (Fig. 11A and B). In contrast, the level of cell surface expression of gB in cells infected with HSV-2 YK811

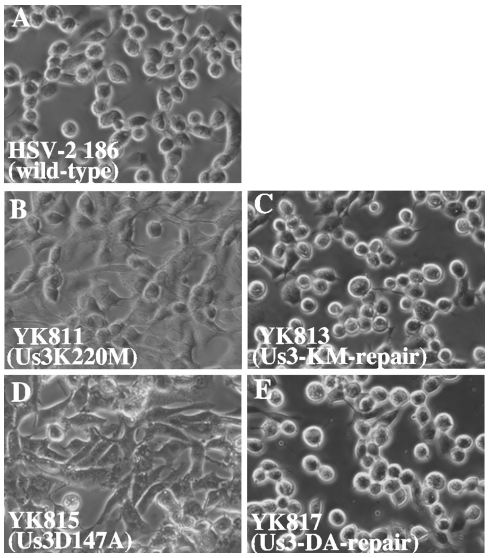

FIG. 8. Digital confocal microscope images of CPEs in Vero cells infected at an MOI of 3 with wild-type HSV-2 186 (A), YK811 (Us3K220M) (B), YK813 (Us3-KM-repair) (C), YK815 (Us3D147A) (D), and YK817 (Us3-DA-repair) (E). Live cells were examined at 24 h postinfection by confocal microscopy. Differential interference contrast images are shown.

(Us3K220M) was almost identical to that in wild-type HSV-2 186-infected cells (Fig. 11C and D).

Taken together, these results indicated that, unlike HSV-1 Us3, HSV-2 Us3 kinase activity was not required for proper localization of UL34 and UL31 proteins, efficient nuclear egress of nucleocapsids, or downregulation of cell surface expression of gB.

Role of HSV-2 Us3 kinase activity in virulence in mice. To investigate the role of HSV-1 and HSV-2 Us3 kinase activities in pathogenesis in vivo, mice were inoculated intracerebrally with various doses of wild-type HSV-1(F), HSV-1 YK511 (Us3K220M), HSV-1 YK513 (Us3-KM-repair), wild-type HSV-2 186, HSV-2 YK811 (Us3K220M), or HSV-2 YK813 (Us3-KM-repair) and monitored daily for mortality. As shown in Table 2, there was a 680-fold decrease in virulence of HSV-1 YK511 (Us3K220M) compared to YK513 (Us3-KM-repair), indicating that HSV-1 Us3 kinase activity played a critical role in virulence in mice following intracerebral inoculation. We noted that there was a 10-fold decrease in virulence of HSV-1 YK513 (Us3-KM-repair) compared to wild-type HSV-1(F),

TABLE 2. LD_{50} values of wild-type HSV-1, HSV-2, and recombinant viruses in mice after intracerebral inoculation

Virus	LD_{50} (PFU)[a]
HSV-1(F)	10
YK511 (Us3K220 M)	68,000
YK513 (Us3-KM-repair)	100
HSV-2 186	0.46
YK811 (Us3K220 M)	10
YK813 (Us3-KM-repair)	1.46

[a] Three-week-old female ICR mice were inoculated with serial 10-fold dilutions of each virus in groups of six per dilution and monitored for 21 days. The LD_{50} values were determined by the Behrens-Karber method.

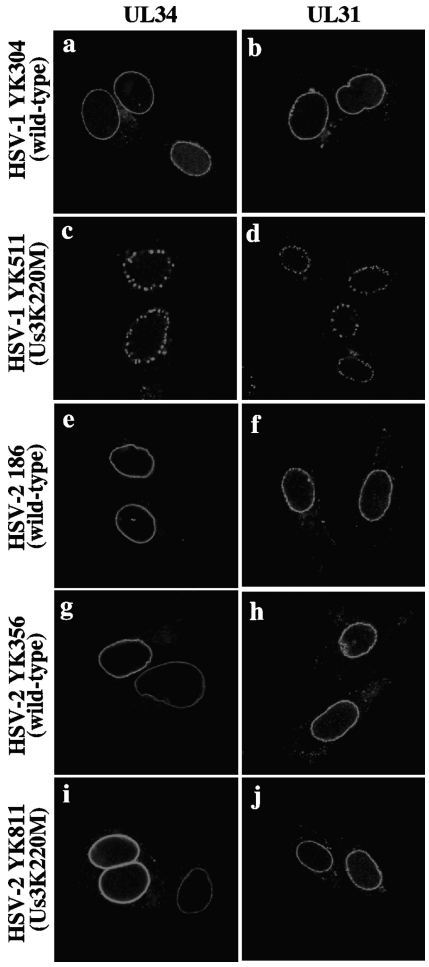

FIG. 9. Digital confocal microscope images showing localization of UL34 and UL31 in Vero cells infected with YK304 (a and b), YK511 (c and d), HSV-2 186 (e and f), YK356 (g and h), and YK811 (i and j) at an MOI of 3. At 12 h postinfection, infected cells were fixed, permeabilized, and immunostained with rabbit polyclonal antibody to UL34 (left panels) or UL31 (right panels) and detected with Alexa-546 conjugated anti-rabbit immunoglobulin G antibody.

suggesting that YK511 and/or YK513 contain a secondary mutation(s) other than K220M in Us3. With regard to HSV-2, there was approximately a sevenfold decrease in virulence of HSV-2 YK811 (Us3K220M) compared to YK813 (Us3-KM-repair) (Table 2). These results indicated that, like HSV-1 Us3, the kinase activity of HSV-2 Us3 played a role in virulence in mice following intracerebral inoculation, but the contribution of HSV-2 Us3 kinase activity to virulence seemed to be much less (100-fold) than that of HSV-1 Us3.

DISCUSSION

The comparative analyses of HSV-1 and HSV-2 Us3 proteins using a genetic manipulation system of HSV-2 with our newly constructed HSV-2 BAC clone in the present study detected significant differences between HSV-1 and HSV-2 Us3 kinases. Overall, it appeared that certain functions that have been attributed to HSV-1 Us3 may not be carried out by HSV-2 Us3 as follows.

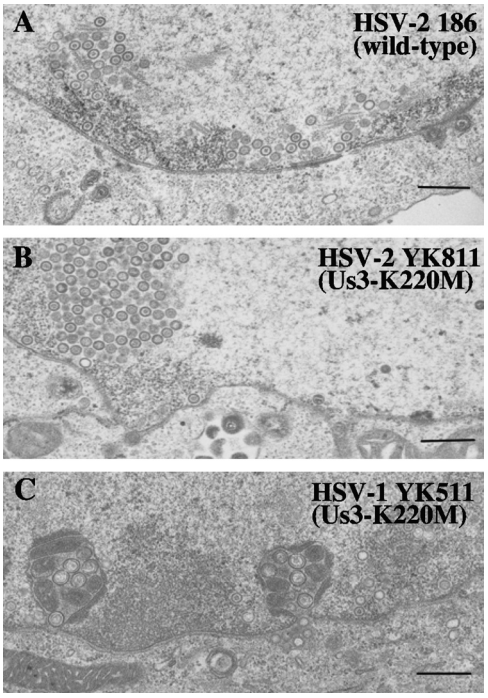

FIG. 10. Electron microscopy of Vero cells infected with wild-type HSV-2 (A), HSV-2 YK811 (Us3K220M) (B), or HSV-1 YK511 (Us3K220M) (C) at an MOI of 5 for 18 h. Scale bars, 500 nm.

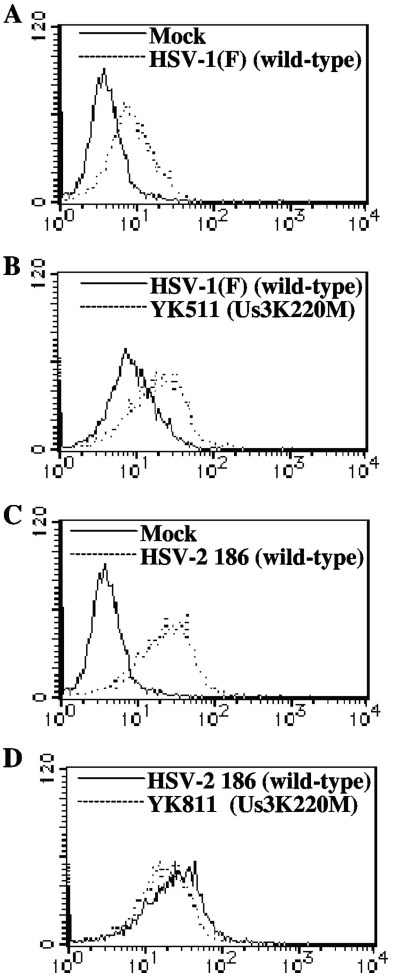

FIG. 11. Detection of gB molecules on the cell surface of infected cells by flow cytometry. Vero cells were mock-infected or infected with wild-type HSV-1(F), HSV-1 YK511 (Us3K220M), wild-type HSV-2 186, or HSV-2 YK811 (Us3K220M) at an MOI of 3. At 6 h postinfection, infected cells were incubated with anti-gB antibody and then analyzed by flow cytometry. (A to D) Cell surface expression of gB in cells infected with one of the two indicated viruses.

It has been well established that one of the major functional consequences of HSV-1 Us3 kinase activity is to promote nuclear egress of nucleocapsids, probably by regulating proper localization of nuclear egress factors UL31 and UL34 at the nuclear membrane (38–40), and this function is conserved in pseudorabies virus (18, 19), another alphaherpesvirus. In the absence of HSV-1 Us3 kinase activity, virions accumulate within distensions of the perinuclear space into the nucleoplasm and the nuclear egress factor UL31/UL34 complex is incorrectly localized at the nuclear membrane, from a smooth pattern to discrete foci that accumulate adjacent to the nuclear membrane distensions in infected cells (40). Mou et al. (29) recently identified UL31 as a Us3 substrate critical for Us3 regulation of nuclear egress of nucleocapsids based on the observation that the Us3 nuclear egress phenotype, including incorrect localization of UL31 and UL34 and virion accumulation in distensions of the perinuclear space, could be mimicked by mutations in Us3 phosphorylation sites in UL31. Surprisingly, although the Us3 phosphorylation sites in HSV-1 UL31 are conserved in HSV-2 UL31 (4, 23), we have shown here that, in the absence of HSV-2 Us3 kinase activity, incorrect localization of UL31 and UL34 proteins at the nuclear membrane and virion accumulation in distensions of the perinuclear space were barely detected. We concluded from these results that the regulatory functions of HSV-1 Us3 in the virion nuclear egress pathway were not conserved in HSV-2 Us3.

Another difference between HSV-1 and HSV-2 proteins observed in the present study was the regulatory effect of Us3 on the cell surface expression of gB. This difference was predictable because the Us3 phosphorylation site in gB (Thr-887) is not conserved in HSV-2 (12). Taken together, these results suggested that the Us3 functions detected only in HSV-1 (including regulation of nuclear egress of nucleocapsids, localization of UL31 and UL34, and cell surface expression of gB) were nonessential in the HSV life cycle, whereas the conserved functions (including regulation of morphology of infected cells and induction of posttranslational modification of HDAC2) were essential in the HSV life cycle. However, we note that HSV-2 may have evolved a viral protein(s) other than Us3 with the regulatory functions of HSV-1 Us3 that have not been conserved in HSV-2 Us3.

We have also shown here that both HSV-1 and HSV-2 with kinase-inactive Us3 exhibited attenuated virulence in mice after intracerebral inoculation. These results indicated that phosphorylation event(s) mediated by HSV Us3 kinase activities contribute to HSV virulence in vivo. It is interesting that the effect of Us3 kinase activity on virulence in mice after intracerebral inoculation appeared to be significantly different be-

tween the HSV serotypes, in agreement with earlier studies using HSV-1 and HSV-2 Us3-null mutant viruses (25, 33). HSV-2 Us3 was apparently less functional in virulence in mice following intracerebral inoculation than HSV-1 Us3. These results were consistent with the observations described above that HSV-2 Us3 had fewer functions than HSV-1 Us3 in infected cells. Thus, it seemed likely that HSV-2 Us3 is much less important for viral replication and pathogenesis than HSV-1 Us3. Further studies, such as investigation of the linkage of HSV-1 and HSV-2 Us3 differences with their virulence in vivo and identification of additional differences between HSV-1 and HSV-2 Us3 kinase functions, are currently under way in this laboratory. Such information may provide insights into the molecular basis of pathological differences between HSV-1 and HSV-2.

Amino acid substitution of a phosphorylation site with an acidic amino acid can be used to investigate the biological role of the phosphorylation site, because such a substitution can mimic constitutive phosphorylation (12, 29, 46). This type of amino acid substitution might occur during evolution of a phosphorylation site in ortholog proteins of different species. However, direct evidence to support this hypothesis has not been reported. In our previous studies of the role of the HSV-1 Us3 autophosphorylation site (Ser-147) in upregulation of its own kinase activity, viral replication, and HSV-1 pathogenesis, we noticed that the HSV-2 Us3 amino acid residue corresponding to HSV-1 Us3 Ser-147 was an acidic amino acid (Asp-147) (13, 41). This led us to hypothesize that HSV-2 Us3 kinase activity is constitutively activated by the presence of Asp-147. In support of this hypothesis, we have shown here that HSV-2 Us3 Asp-147 was essential for its intrinsic kinase activity. Although we should note that further studies, including replacement of Asp-147 region in HSV-2 Us3 with a Us3 phosphorylation site and a demonstration that HSV-2 Us3 activity is regulated by phosphorylation at the site, are required to show that Asp-147 of HSV-2 Us3 is functionally equivalent to phosphorylated Ser-147 of HSV-1 Us3, these observations raised an interesting possibility that the autophosphorylation site in Us3 evolved in HSV-1 to tightly regulate its kinase activity, while Us3 Asp-147 evolved in HSV-2 to activate its kinase activity constitutively. Similarly, it has been reported that the phosphorylation site Ser-244 in human cellular tight junction protein ZO-2 is not conserved in mouse and rat ZO-2 orthologs and the orthologous residues for Ser-244 in mouse and rat ZO-2 are Asp (3; www.phosphosite.org/siteAction.do?id=25048), although the function of Ser-244 phosphorylation in human ZO-2 and its orthologous residues in mouse and rat ZO-2 has not been determined. In addition, an identical amino acid substitution (S→D) was also detected in the orthologous residues of the phosphorylation site (Ser-1124) of human splicing factor SRm300 and its rat and mouse orthologs (3; www.phosphosite.org/siteAction.do?id=16915). These results suggested that the phosphorylation site in a kinase substrate might sometimes evolve from an acidic amino acid in orthologs of other species or vice versa, thereby changing the regulatory function of the site. These observations may provide a new insight into the evolution of protein kinases and their substrates. Furthermore, the regulatory role of the orthologous residue of HSV-1 Us3 Ser-147 in HSV-2 Us3 elucidated in the present study reinforced the significance of HSV-1 Us3 Ser-147 in regulation of its own kinase activity, as reported previously (13, 41).

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