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Genetic Engineering of a Modified Herpes Simplex Virus 1 Vaccine Vector

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Abstract

The herpes simplex virus 1 (HSV-1) *d*106 mutant virus is a multiple immediate-early gene deletion mutant virus that has been effective as an AIDS vaccine vector in rhesus macaques (Kaur et al. 2007. Virology 357: 199–214). Further analysis of this vector is needed to advance development into clinical trials. In this study we have defined the precise nature of the multiple IE gene mutations in the *d*106 viral gene and have used this information to construct a new transfer plasmid for gene transfer into *d*106. We tested the effect of an additional mutation in the U_L41 gene on *d*106 immunogenicity and found that it did not improve the efficacy of the *d*106 vector, in contrast with results from other studies with U_L41 gene mutants. The safety profile of *d*106 was improved by generating a new vector strain, *d*106S, with increased sensitivity to acyclovir. Finally, we have constructed a *d*106S recombinant vector that expresses the HIV clade C envelope protein. The *d*106S-HIVenvC recombinant has retained the sensitivity to acyclovir, indicating that this phenotype is a stable property of the *d*106S vector.

Introduction

Viral vaccines were historically either inactivated viruses or live, attenuated viruses [1], but these types of vaccines have not been feasible for certain viruses such as the herpes viruses or HIV. Therefore, new types of vaccines including plasmid DNA vectors and replication-defective mutant viruses have been investigated. Replication-defective mutant viruses are genetically engineered or spontaneous mutant viruses that are defective for a viral function essential for replication in normal cells, but that can replicate in cells that express the missing viral gene product [2]. Replication-defective mutant viruses have been considered for vaccines for smallpox [3,4], genital herpes [5] and AIDS [6]. Similarly, replication-defective mutant adenoviruses [7], poxviruses [8], and alphaviruses [9] have been studied as vaccine vectors. Adenoviruses have been tested extensively as AIDS vaccine vectors [10], but the failure of the recent STEP trial utilizing Ad vectors [11] raises the need for additional vector approaches.

We have constructed replication-competent and replication-defective HSV-1 recombinant viruses that express SIV gene products and have used these to immunize rhesus macaques. The

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initial recombinants expressed a number of HSV gene products in addition to SIV proteins and induced SIV-specific humoral and cellular immune responses that resulted in partial protection of macaques against mucosal SIVmac239 challenge infection [12]. A second-generation HSV vector, HSV-1 *d*106, has multiple IE gene mutations; thus, in normal cells it expresses only two viral gene products, ICP0 and ICP6 [13]. HSV-1 *d*106 causes minimal host protein shutoff, and minimal cyopathic effect, and shows prolonged expression of a transgene in infected cells [14]. HSV-1 *d*106 recombinants expressing SIV env, gag, and a rev-tat-nef fusion protein were constructed [14] and used to immunize rhesus macaques [15]. Immunized macaques showed reduced viral loads, which correlate with certain cellular and humoral immune responses [15]. Based on these results and those of further studies (Kaur et al., unpublished results), we are developing *d*106 recombinants as AIDS vaccine vectors for clinical trials. In this study we have defined the IE gene mutations in *d*106, constructed a new plasmid transfer vector, tested the effect of mutating the virion host shutoff function (vhs) on immunogenicity, and improved the safety profile of the vector strain by making it more sensitive to a herpes antiviral drug, acyclovir.

Results

Sequencing of IE gene mutations in the *d*106 genome

The *d*106 mutant virus [13] was constructed to have mutations in the *ICP4* and U_L54 ORF encoding *ICP27* and in the intergenic regions of the promoter/enhancer between the *ICP4* and *ICP22* ORFs and between the *ICP4–ICP47* ORFs (Figure 1C). Because *d*106 is being developed as a vaccine vector [14,15] for clinical trials, it is necessary to determine the exact sequence of all deletions and mutations in the vector strain. Therefore, we sequenced the mutations and GFP cassette insertion in the *d*106 strain by direct sequencing of *d*106 genomic DNA.

In the original construction of the *d*106 mutant virus [13], the CMV-GFP cassette was inserted in the *ICP27/*U_L54 gene between the *Bam*HI (nts 113322–113327) and *sa*I (nts 114517–114522) sites (Figure 1C). This deletion inactivates the essential *ICP27/*U_L54 gene, making *d*106 replication dependent on ICP27/U_L54 expressed from the gene in the complementing cell line. Our sequencing results confirmed that nts 113328–114516 were replaced by the CMV-GFP cassette, between the *Bam*HI and *SaI*I restriction endonuclease cleavage sites (Figure 2A). In addition, the CMV-GFP expression cassette was inserted in the opposite orientation to the U_L54 ORF and flanked on the left by one copy of the *PacI* linker (TTAATTAA) and on the right by three copies of the *Pac* linker, and this entire cassette was bounded by *BbaI* sites (Figure 2A). Therefore, the deleted sequences, the orientation of CMV-GFP cassette insertion, and linker sequences are totally consistent with the original transfer plasmid and virus construction [13].

The *ICP4* open reading frame deletions in *d*106 virus (Figure 1C) were isolated originally in the *d*120 mutant virus and estimated to be 4.12– 4.15 kbp each [16], but the exact size of the deletions had not been defined. This deletion inactivates both copies of the essential *ICP4* gene, making *d*106 virus replication dependent on ICP4 provided by the complementing cell line. Our sequencing results identified a 4222 bp deletion of nts 126371–130592 (Figure 2B) and 147641–151862. These deletions represent 3361 bp deletion from each of the two *ICP4* ORFs, leaving 535 bp or 178 codons in the 5′ end of the *ICP4* ORF followed by a frame-shift.

*d*106 was also constructed to have 270 bp deletions in the S component repeated sequences in the promoters between the *ICP4* and *ICP22* genes and between the *ICP4* and *ICP47* genes [13]; Figure 1C). These deletions remove an Oct-1 site, effectively making the *ICP22* and *ICP47* genes into early genes whose expression is dependent on ICP4 provided by the complementing cell line [13]. Our sequencing results showed that a 265 bp sequence between

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EcoR I and *BssH* II restriction enzyme sites from nts 131,539–131,803 and 146429–146695 was replaced by a 16-bp sequence, GCTCTAGATTAATTAA, used as a linker in the construction of the TGTΔ mutation (Figure 2C). The 265 bp of deleted sequence contains three SP1 binding sites and a TTAATGARAT Oct-1 site within the promoter of the *ICP22* and *ICP47* genes from nts 131536–131543, nts 131685–131693, and nts 131786–131794. Deletion of the SP1 and Oct-1 sites is believed to result in the disruption of promoter activity and the loss of the transcription of the *ICP4*, *ICP22*, and *ICP47* genes.

The mutations in the *d*106 viral genome were confirmed by sequencing of another *d*106-derived recombinant, *d*106-27lacZ [14]. This virus was constructed by the replacement of the GFP cassette of ICP27/U_L54 region of *d*106 with a *lacZ* cassette using homologous recombination by co-transfection of *d*106 genomic DNA and pd27-bgal plasmid DNA, which has HSV sequences from nts 112700–113326 (625 bp) and nts 114591–115957 (1366 bp) flanking the *lacZ* expression cassette. The IE gene mutations in *d*106-27lacZ viral DNA were sequenced and found to contain the same sequence alterations as *d*106 described above.

Derivation of a new transfer plasmid

Co-transfection of infectious HSV DNA with linear viral DNA sequences into cells to allow homologous recombination is one approach used to introduce new sequences into the viral genome [17], and this approach has been used to introduce expression cassettes into the HSV genome to generate vaccine vectors [12,14]. We had used the pPs27pd1 plasmid (Figure 3) to introduce a *lacZ* expression cassette into the *ICP27/U_L54* locus of *d*106 to construct *d*106-27lacZ, as described above. This transfer plasmid has proven useful, but the recombination efficiency was low, possibly due to its short HSV sequences flanking the expression cassette (only 625 bp on one side). Therefore, we constructed a new transfer plasmid by amplifying two 1.3 kbp flanking sequences from *d*106 viral DNA, one from nts 112000–113303 (1324 bp) and another from nts 114541–115860 (1320 bp). Two DNA fragments from nts 112000–113303 (F1) and nts 114541–115860 (F2) were PCR-amplified from *d*106 DNA using primer pairs A and B, respectively (Table 1). The PCR fragments were gel purified and ligated with TA plasmid DNA. The F1 insert was removed from TA-F1 by *EcoR* I and *Hind* III digestion and inserted into pUC19 plasmid between the *EcoR* I and *Hind* III sites to construct the pUC19-F1 plasmid. The F2 insert was removed from TA-F2 by *EcoR* I digestion and inserted into the EcoR I site of pUC19-F1 to construct pUC19-F2-F1. The CMV immediate-early promoter/enhancer-multi-cloning site – SV40 polyadenylation signal [CMV-MCS-poly (A)] cassette was removed from pCIΔ*AfI* III [12] by *BgI* II and *Bam*HI digestion and ligated into the p54–53 plasmid cleaved with *BgI* II and *Bam*H I and filled in with Klenow to generate the pd27B plasmid. The new pd27B plasmid (Figure 3) and used as the transfer plasmid for the construction of a recombinant virus expressing HIV clade C envelope, as described below, and other microbial antigens (R. Colgrove and D.M. Knipe, in preparation).

Effect of vhs inactivation on immunogenicity of *d*106 vectors

Previous studies have reported that inactivation of the vhs function can increase immunogenicity of HSV strains in some situations [18–20], although not all situations [21]. We wanted to determine if a *vhs* mutation could increase immunogenicity of a recombinant *d*106 viral vector expressing HIV gag. We first established a quantitative ELISPOT assay for $CD8^+$ T cells specific for HIV gag. We constructed a *d*106-HIVgag recombinant virus by insertion of an HIV gag expression cassette into *d*106 as described in Materials and Methods. This recombinant expressed gag protein for at least 36 hpi in Vero cells (Figure 4A). We immunized groups of mice (n=6) with 5×10^2, 5×10^4, or 5×10^6 PFU of *d*106-HIVgag at days 0, 21, and 42. Splenocytes were collected from each recombinant-infected mice (three mice per group per time point) at 7 days after each boost and were stimulated with an MHC-I gag peptide. The gag-specific $CD8^+$ T cell responses were proportional to the dose of vector

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inoculated (Figure 4B). Furthermore, the $CD8^+$ T cell responses were higher after the priming immunization (4 weeks) than after the boosting immunization (7 weeks) (Figure 4B). Therefore, this system provided a quantitative assay for comparing the T cell responses to different *d*106 constructs.

We then constructed a vector lacking *vhs* by inserting the HIV gag expression cassette into a vhs-deleted, *d*106-derived recombinant virus named RJ-1 [22], as described in Materials and Methods. The RJ-1 HIVgag recombinant expressed slightly less gag protein than the *d*106-gag recombinant (Figure 4A). We infected mice with the *d*106-HIVgag and RJ-1-HIVgag recombinants and measured $CD8^+$ T cell responses by ELISPOT assays and antibody responses by ELISAs. In contrast with previous results with *vhs* mutant viruses, the RJ-1 HIV gag virus induced lower $CD8^+$ gag-specific T cell responses (Figure 5A) and lower p24-specific antibody responses than the *d*106 vector (Figure 5B). HSV-specific antibody responses were similar for the two vectors (Figure 5C). These results indicated that mutation of *vhs* yields no improvement in immunogenicity in the context of the *d*106 mutant virus.

Acyclovir resistance of and engineering of a sensitive *d*106S strain

In our studies of the properties of the HSV-1 *d*106 virus, we observed that *d*106 virus was partially resistant to a herpes antiviral drug, acyclovir, in having a 50% inhibitory concentration (IC_{50}) of 146 μM, as compared with the parental virus, HSV-1 KOS, which showed an IC_{50} of 13 μM (Figure 6). For an optimal safety profile, an HSV vector should be sensitive to acyclovir, so we back-crossed *d*106 with KOS WT virus to generate a new version of *d*106 that was acyclovir-sensitive. For the back-cross, we co-infected E-11 complementing cells with KOS and *d*106 viruses at an MOI of 3 and harvested the progeny virus. The resulting progeny virus stock was used to infect E-11 cells at high dilutions so that well-isolated viral plaques were formed. Green fluorescent plaques were picked and screened for sensitivity to 30 μM acyclovir and 60 μM acyclovir. Four of 140 plaque isolates showed sensitivity to 30 μM acyclovir and were further plaque-purified. These 4 isolates were screened for growth on E-11 cells containing the *ICP4* and *ICP27/U_L54* genes, V827 cells containing the *ICP27/U_L54* and *ICP8* genes, and Vero cells, the parental cell line containing no HSV genes. One plaque isolate with high sensitivity to acyclovir formed green plaques on E11 but not on Vero and V827 cells, and this virus was designated as *d*106S. The acyclovir sensitivity of *d*106S was assayed in comparison with KOS and *d*106 (Figure 6). The results showed that the IC_{50} for *d*106S was 6.6 μM, about 2-fold lower than KOS and 20-fold lower than *d*106, indicating that we had generated an acyclovir-sensitive vector strain, *d*106S.

To ensure that the *d*106S virus contained all of the mutations engineered into *d*106, we sequenced the regions of mutations in *d*106S. Our sequencing results confirmed that *d*106S contained the GFP insertion in the *ICP27/U_L54* gene, the deletions in the *ICP4* gene, and the deletions within promoter regions of the *ICP22* and *ICP47* genes (data not shown). Therefore, we concluded that *d*106S contains the five IE gene deletions in the original *d*106 virus but has high sensitivity to acyclovir, suggesting that *d*106S has WT *PoI* and *TK* genes, which define acyclovir sensitivity.

Construction of *d*106S recombinant viruses expressing HIV envelope protein

We wanted to use the HSV-1 *d*106S virus as a vector for expression of HIV gene products to serve as a candidate for a clinical trial vector. As the first application of this vector, we constructed a *d*106S recombinant virus that expresses the HIV clade C envelope (env) protein, *d*106S-HIVenvC, using homologous recombination.

The transfer plasmid was constructed as follows. Plasmid pVRC5309, which contains an expression cassette with a codon-optimized HIV (Clade C) envelope protein (envC) ORF, was

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digested with *BamH* I and *Not* I, and the resulting 1880 bp fragment containing the envC ORF was inserted into the pd27B plasmid digested with XbaI and NheI and filled with Klenow. The resulting plasmid, designated pd27B-5309, was linearized by *Swa* I digestion and co-transfected with *d*106S viral DNA into E-11 cells. Viral progeny were harvested, and recombinants were identified in a screen for non-fluorescent plaques, because homologous recombination leads to the incorporation of the CMV-env cassette into the *ICP27/U_L54* gene locus replacing the GFP expression cassette. Three isolates were plaque-purified, and expression of env protein was assessed (Figure 7A). One of the recombinant viruses was chosen as the prototype strain and was shown to express env protein for at least 24 hours (Figure 7B). Furthermore, the recombinant virus was highly sensitive to acyclovir (Figure 7C) with an IC_{50} of 6.1μM. The *d*106S-HIVenvC recombinant viruses expressed the env protein, and retained the acyclovir sensitive property of *d*106S. Therefore, the acyclovir-sensitivity of the modified vector, *d*106S, is stable through construction of new recombinant strains.

Discussion

The HSV-1 *d*106 recombinant virus has shown good immunogenicity and protective capacity in a rhesus macaque model of SIV infection [15], and we are developing this virus as an AIDS vaccine vector for clinical trials. In this study we have determined the precise nature of the multiple IE gene mutations in the *d*106 viral genome and have used this information to generate a transfer plasmid containing larger flanking sequences for improved efficiency of gene transfer into recombinant virus vectors. Previous studies with HSV mutants have shown increased immunogenicity with viral strains that have the *U_L41* gene mutated and virion host shutoff function inactivated [18,19], but we observed slightly lower expression of the HIV gag protein from a vector lacking U_L41 and reduced cellular and humoral immunogenicity. Finally, we improved the safety profile of *d*106 for clinical use by generating a modified virus, *d*106S, that shows increased sensitivity to acyclovir.

Improved transfer plasmid

Previous studies have shown that the efficiency of gene transfer is proportional to the length of the sequences in the donor DNA [17]. We therefore used our data on the sequences surrounding the *ICP27/U_L54* gene deletion/insertion in *d*106 to make a new transfer plasmid, pd27B, with longer flanking sequences around the transgene site than the plasmid pPs27pd1, which we had previously used to insert transgenes in the *ICP27*/U_L54 gene [14]. The plasmid pd27B contains flanking sequences of 1324bp and 1320 bp as compared with 626 bp and 1366 bp for pPs27pd1. The increased flanking sequences in pd27B have resulted in a higher frequency of recombination and greater ease of making recombinants (results not shown).

ACV sensitivity

HSV recombinants delivered for clinical purposes need to be susceptible to standard anti-HSV drugs so that the drugs, in the unlikely event that any revertant or recombinant that might arise, could control this virus. It is highly unlikely that a revertant could arise because the *d*106 strain has multiple deletion mutations in genes encoding functions that cannot be complemented by cellular gene products. It is also unlikely that the mutant virus could re-gain the missing viral genes from the complementing cell line due to a lack of homologous sequences for recombination. The missing functions cannot be provided by another herpesvirus, except for HSV-1 and HSV-2. However, a drug resistance mutation could be transferred from the vaccine strain to a naturally occurring HSV-1 or HSV-2 strain by homologous recombination. Therefore, it is essential that HSV strains used for clinical applications be sensitive to antiviral drugs. We observed that the *d*106 virus was moderately resistant to acyclovir so we backcrossed it with WT KOS virus to obtain a *d*106-derived strain, *d*106S, with acyclovir-sensitivity similar to the WT KOS parental strain. The acyclovir sensitivity phenotype was maintained in *d*106S

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after construction of an HIV gag-expressing recombinant, indicating that this is a stable property of *d*106S.

Lack of an effect of vhs on *d*106 immunogenicity

The HSV *U_L41* gene encodes the virion host shutoff function, a function which reduces host mRNA translation in HSV-infected cells [23]. The U_L41 protein is incorporated into the tegument layer of the virion and when delivered into the cytoplasm is activated to degrade mRNAs on polyribosome. Inactivation of the *U_L41* gene and loss of the vhs function increases immunogenicity of certain virus strains [19,20,24] so it was surprising that this additional mutation did not increase immunogenicity of *d*106. This is likely explained by the fact that *d*106 already shows a defect in shutoff of host translation because of the mutation in the *ICP27/U_L54* gene. Shutoff of host protein synthesis by HSV requires both vhs (*U_L41*) and ICP27 (*U_L54*) [25]. ICP27/UL54 inhibits splicing of host mRNA [26] and cell gene transcription [27], so it is also required for host shutoff. The vhs function is required for the ability of HSV to block dendritic cell activation [28]. Because either *ICP27/U_L54* or *U_L41* gene inactivation seems to enhance immunogenicity of HSV vaccine vectors and both are required for shutoff of host protein synthesis, the increased immunogenicity is likely due to a lack of host shutoff, and not other effects of vhs on dendritic cell function or innate responses [29].

Comparisons of different HSV vectors

In addition to replication-defective mutant HSV strains, HSV amplicons have been constructed that express the HIV gag protein [30]. These amplicons contain no known HSV ORF and express no HSV proteins. Immunogenicity studies using amplicons in mice have been published [30,31], but thus far no studies of immunogenicity in monkeys have been published. Recent studies have shown that the immediate-early ICP0 protein is required for chromatin modification and remodeling to allow efficient expression of genes on the viral genome [32, 33]. Therefore, expression of ICP0 by a HSV vaccine vector is likely to prevent host chromatin silencing of the viral genome and enhance trans-gene expression from the viral genome and the immune responses elicited. This hypothesis is consistent with decreased transgene expression by the *d*109 virus, which does not express any IE genes [13].

In summary, we have modified the HSV-1 *d*106 vector system by generating the *d*106S virus strain with increased acyclovir sensitivity. Furthermore, the *d*106S virus strain shows the properties of limited cytopathic effect and prolonged expression of transgenes. We are now constructing and characterizing a set of *d*106S vectors expressing HIV proteins for future clinical trials.

Materials and Methods

Plasmids

The pVRC5309 plasmid [34] containing a codon-optimized HIV clade C envelope protein (envC) ORF and pVRC4302 plasmid [35] containing the HIV gag-pol-nef expression cassette were provided by Dr. Gary Nabel, NIH. The pdl27CIA plasmid was described previously [12]. The TA (PCR 2.1) plasmid was obtained from Invitrogen (Carlsbad, CA).

Cells and viruses

Vero cells, V827 cells containing the *ICP8* and *ICP27* genes [36]) and E-11 cells containing the *ICP4* and *ICP27* genes [13] were cultured as described. The HSV-1 *d*106-LacZ virus [14], the HSV-1 *d*106 virus [13], and the RJ-1 virus (*d*106Δvhs) [22] were described previously. Low passage HSV-1 strain KOS virus was obtained from Dr. Priscilla Schaffer.

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The HSV-1 *d*106-HIVgag and RJ-1-HIVgag viruses were constructed as follows. The gag-pol ORF from the plasmid VRC4302 was removed by *HpaI* and *Eco*RI digestion followed by Klenow treatment to fill in the ends. This fragment was inserted into plasmid pdl27.CIA [12] at a filled- in *AflII* site. A partial digest was performed using *Sgr*AI, allowing the removal of 2484bp of the pol gene (corresponding to base pairs 2334–4818 of HXB2). The vector was ligated using the open *Sgr*AI sites, generating the gag expression cassette. The pdl27 gag vector was linearized with *SwaI*I and cotransfected with *d*106 or RJ-1 viral DNA. Progeny viruses were harvested, and non-fluorescent plaque-forming viruses were purified three times.

Viral DNA Purification and Sequencing

Viral DNA was purified from infected cell lysates by sodium iodide density gradient centrifugation, as described previously [37] except that Proteinase K was used in place of Pronase. For sequencing, the viral DNA was partially digested with *Eco*RV restriction endonuclease, precipitated with ethanol, and analyzed by ABI sequencing at the Dana Farber/Harvard Cancer Center DNA Resource Core using primers based on the strain 17 reference sequence (NC-001806; [38].

Western blotting

Vero or HEp-2 cells were infected with the indicated viruses, and at the times indicated, whole cell lysates were prepared and resolved by SDS-PAGE. Western blot detection of HIV gag or clade C was performed using anti-HIV gag antibody (#4121) or anti-HIV env antibody (#1209) from the NIH AIDS Research and Reagent Program as done previously [14].

Animal procedures

Animal studies were conducted in accordance with National Institutes of Health (NIH) and Harvard University guidelines. Six-week-old female BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and acclimated for one week prior to use. The mice were inoculated by subcutaneous (s.c.) infection in the left flank with the doses of viruses indicated or uninfected Vero cell lysate. The inoculations consisted of 20 μl of lysate stock diluted into sterile, endotoxin-free 0.9% sodium chloride solution (Sigma) per mouse.

Blood samples were collected by retro-orbital plexus puncture, and sera were prepared using Microtainer serum separators (Becton Dickinson) and stored at −20°C until analysis. Enzyme-linked immunosorbent assays (ELISAs) to determine antigen-specific IgG titers were conducted as described previously [39], except that 96-well microtiter plates were coated with HSV-1 viral lysate virions (Advanced Biotechnologies Inc) at 50 ng per well or HIV p24 recombinant protein (Protein Sciences Corp. Meriden, CT) at 200 ng per well.

Elispot assays were performed to measure the mouse $CD8^+$ T cell responses against HIV Gag antigen using a BD ELISPOT Set (BD Biosciences Pharmingen) as described previously [14], except that MHC class I-restricted HIV Gag peptide (AMQMLKETI) was used in this study.

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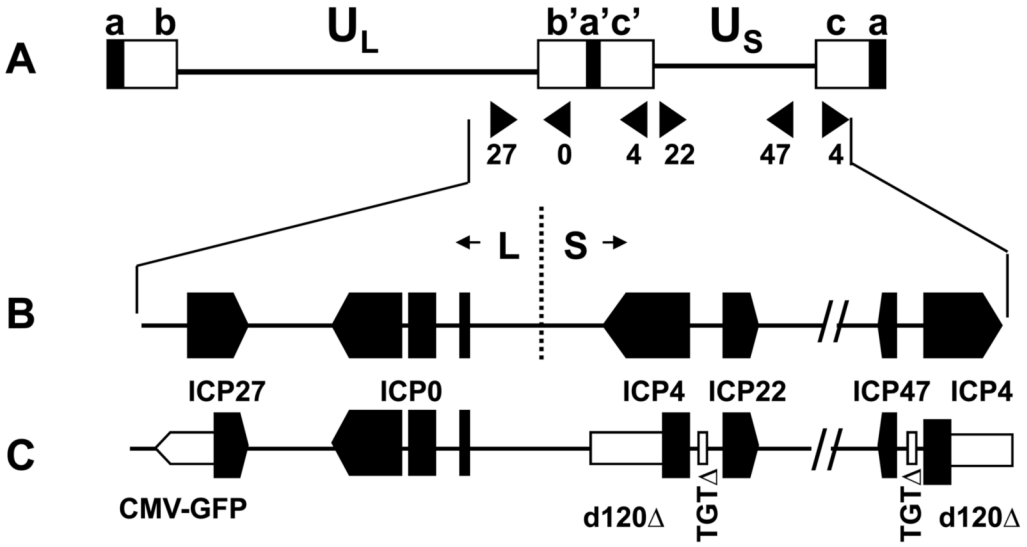

Figure 1. Organization of the HSV-1 genome

A.Diagram of the structure of the HSV genome. The unique sequences are represented as a line, and the repeated sequences are represented as boxes. a= terminal repeats, b, b′ = L component inverted repeats, c, c′= S component inverted repeats; **B.** Expanded map of the right end of the genome showing the ORFs of the IE genes; **C.** Expanded map of the right end of the *d*106 genome showing the deletions (open boxes) of *ICP4* and *ICP22/47* gene promoters, and the CMV-GFP cassette (open arrow) insertion in the *ICP27* gene.

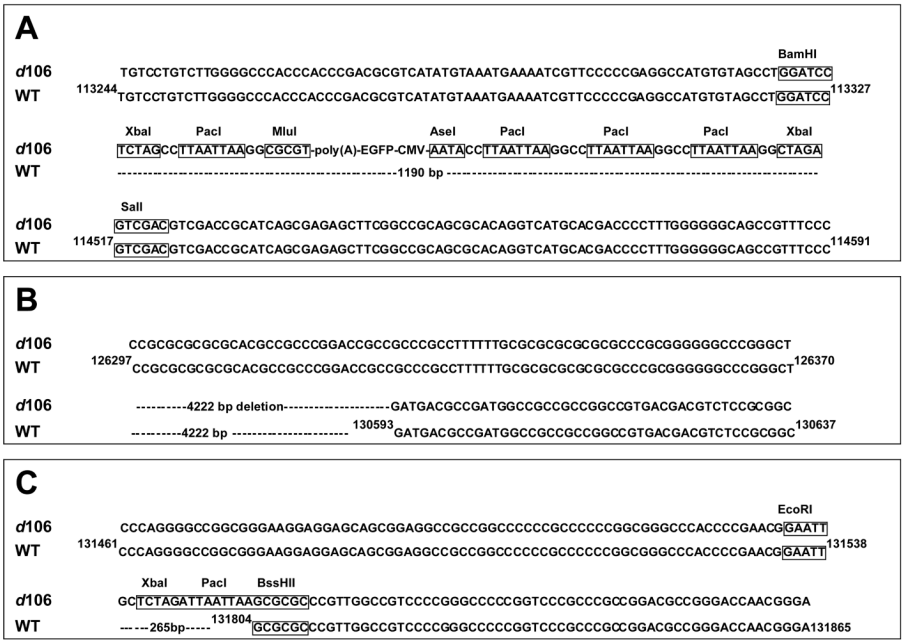

Figure 2. Locations of deletions and GFP insertion in d*106* genome

A. DNA sequence of the HSV sequences flanking CMV-GFP cassette insertion between nt 113327 and nt 114517 of *BamH* I and *SaI* I sites of *ICP27* locus. **B.** *ICP4* gene deletions from nts 126370–130593. The second deletion from nts 147641–151862 is not shown. **C.** Deletions within ICP22/47 promoters from nts 131538–131804 and 146430–146694.

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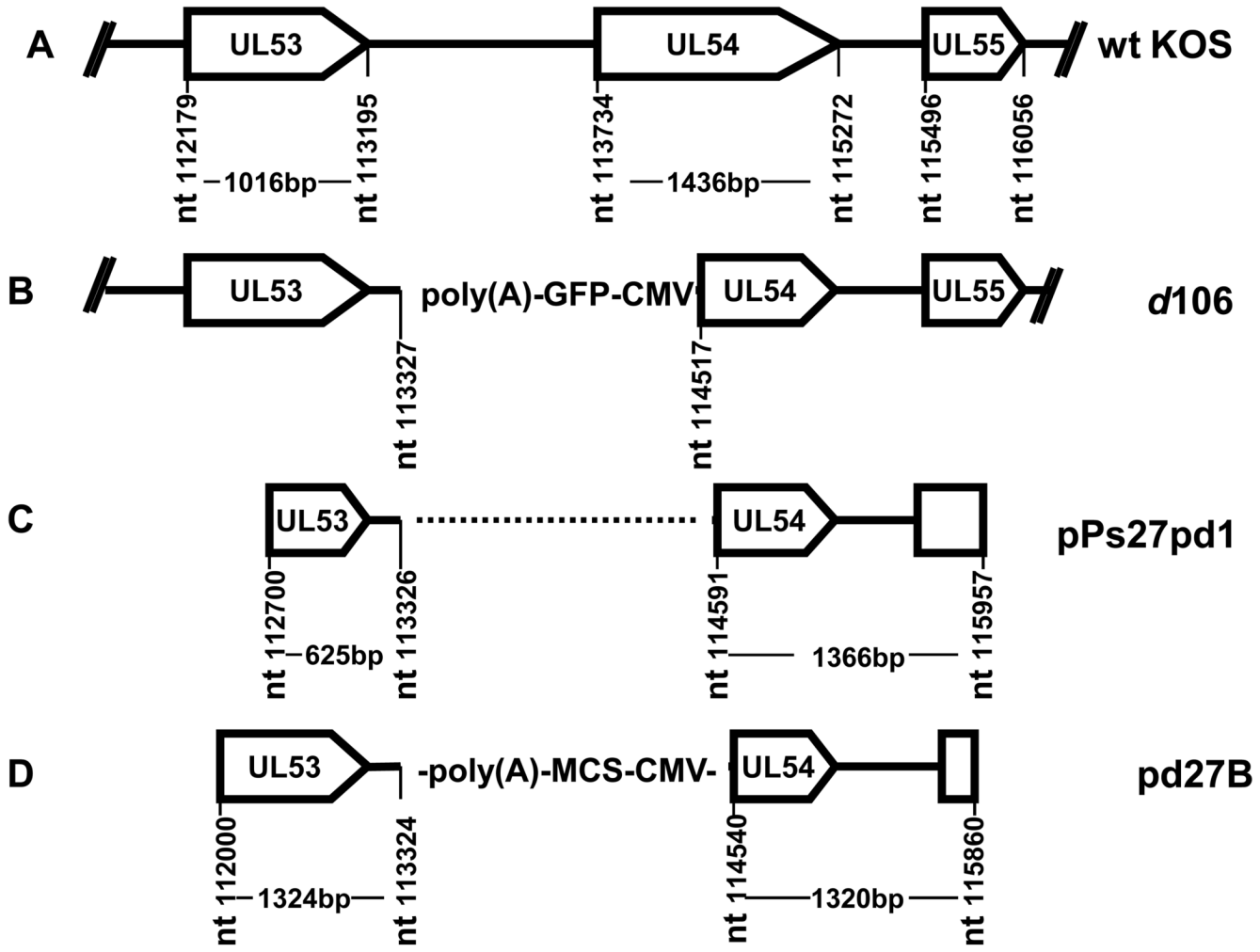

Figure 3. Structure of transfer plasmids

A. Structure of the HSV-1 WT KOS genome from the *U_L53* to *U_L55* genes. **B.** Structure of the HSV-1 *d*106 genome from the U_L53 to U_L55 genes showing the CMV GFP expression cassette inserted between nts 113327–114517. **C.** Structure of the insert in the pPs27pd1 transfer plasmid with KOS DNA fragments from nts 112,700–113,326 and nts 114591–115,957. **D.** Structure of insert in transfer plasmid pd27B containing KOS DNA fragments from nts 11200–113324 and nts 114,540–115,860 flanking the CMV-multiple cloning site-poly(A) cassette.

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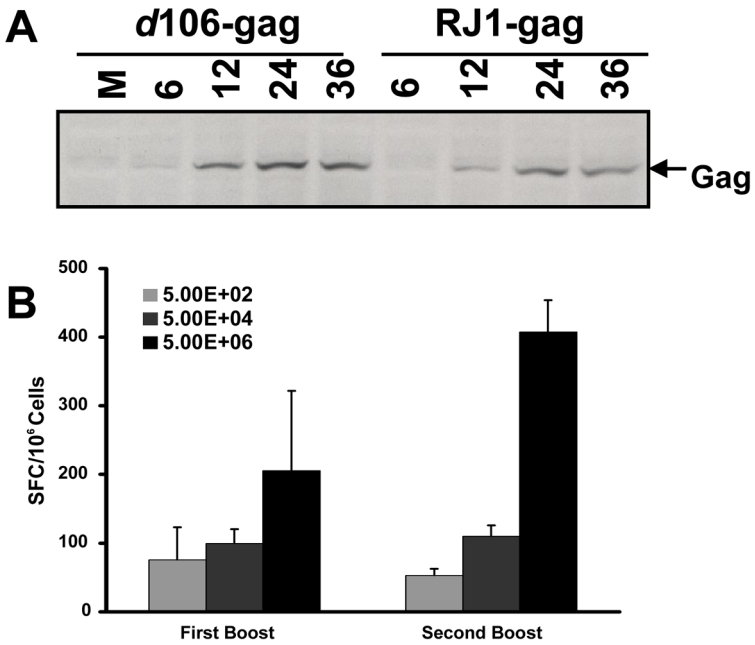

Figure 4. HIV gag expression and CD8$^+$ T cell response induction by HSV recombinants

A. HIV Gag protein expression in Vero cells infected with *d*106-gag and RJ1-gag recombinant viruses at various times post infection (hours), as detected by Western blots. M=mock-infected; **B.** Effect of varying the dose of immunizing virus on HIV Gag specific CD8$^+$ responses. Groups of mice (n=6) were immunized with 5×10^2, 5×10^4, or 5×10^6 PFU *d*106-gag, at days 0, 21, and 42. Splenocytes were collected from each recombinant-infected mice (three mice per group per time point) at 7 days after each boost and were stimulated with an MHC-I Gag peptide. Results are shown as the mean number of interferon-γ spot-forming cells (SFC)/10^6 splenocytes±standard deviation.

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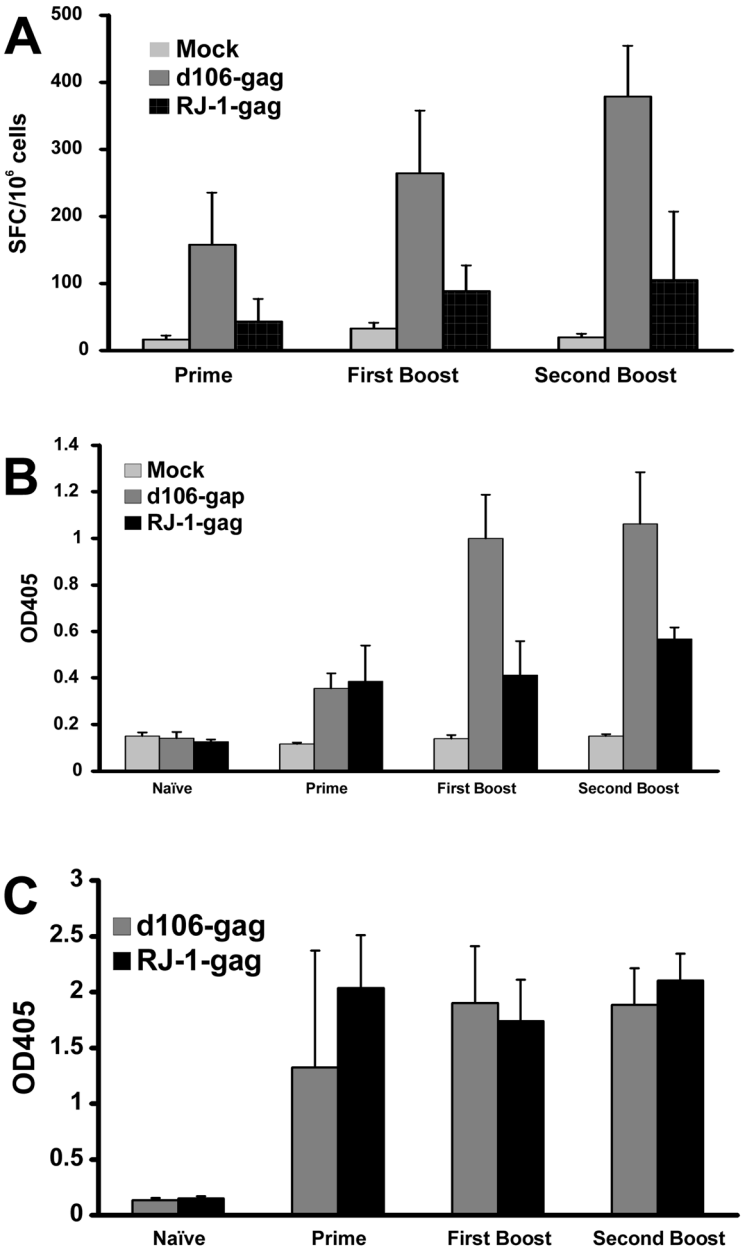

Figure 5. Effect of a vhs mutation on *d*106 immunogenicity

Mice were inoculated with 2×10^6 PFU of *d*106-gag or RJ1-gag virus, followed by two booster inoculations at week 3 and week 6. Splenocytes were collected at week 4 and week 7 for ELISPOT as described in Figure 4. Serum samples were collected at week 0, week 3, and week 6 prior to booster inoculation, and week 9 after booster. **A.** HIV gag-specific $CD8^+$ T cell responses as measured by ELISPOT. **B.** HIV Gag antibody responses, and **C.** anti-HSV antibody responses induced by *d*106-gag and *d*106Δvhs-gag (RJ1-gag) recombinant viruses determined by ELISA. The results are shown as mean of OD_{405}±standard deviation.

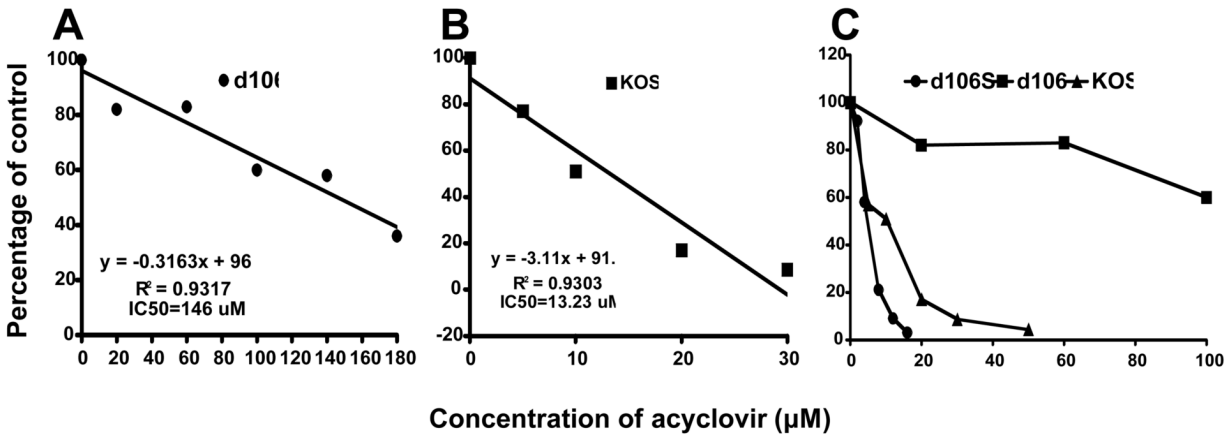

Figure 6. Acyclovir sensitivity of HSV-1 KOS WT, *d*106, and *d*106S viruses

One hundred plaque-forming units (PFUs) of HSV-1 KOS, *d*106 or *d*106S virus were plated on E-11 cells, and the cultures were incubated with the indicated concentrations of acyclovir. The mean number of plaques formed in each concentration of acyclovir was divided by the mean number of plaques in the absence of acyclovir, and the results were expressed as percent of control.

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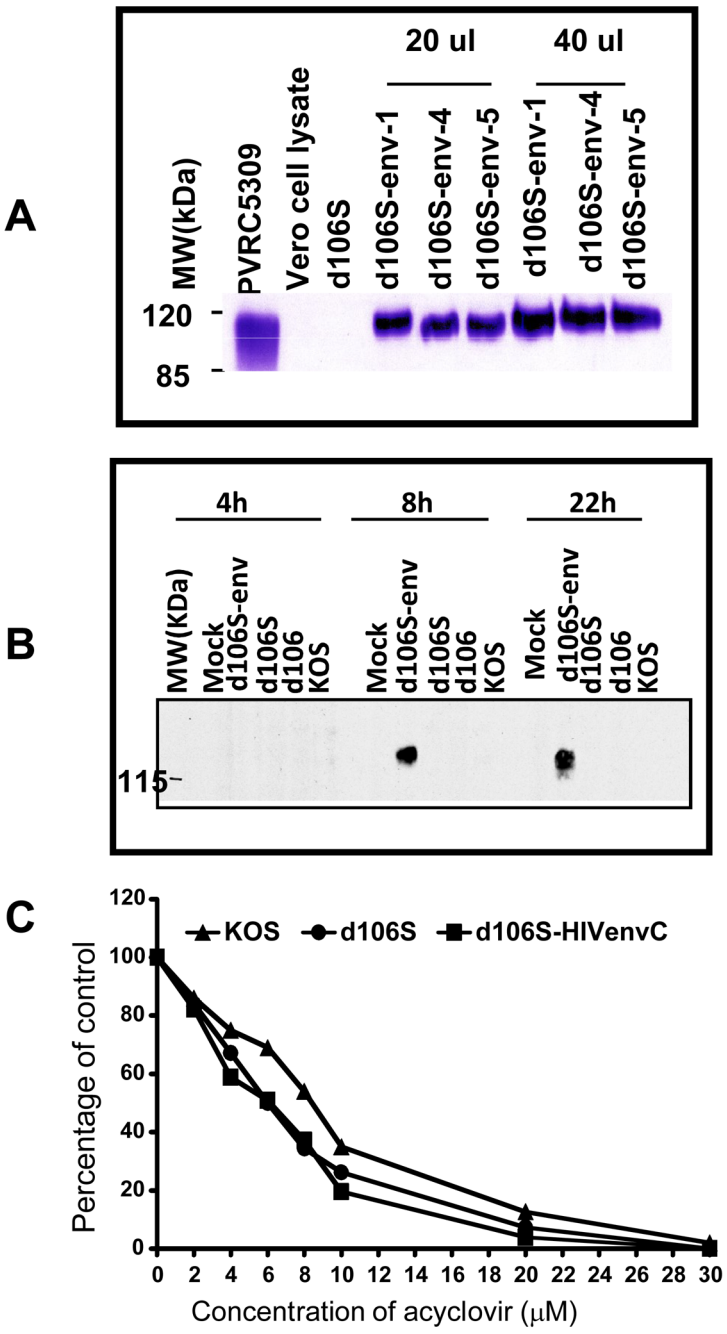

Figure 7. Properties of the *d*106S-HIVenvC viruses

A. Identification of three recombinants (1, 4, and 5) showing HIV Env p120 protein expression in Vero cells at 48 h p.i. by Western blot analysis. **B.** Duration of env expression in HEp-2 cells by *d*106S-envC. **C.** Acyclovir sensitivity of the *d*106S-HIVenvC recombinants.

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Table 1

Primers used in this Study

Primer pair A:

Forward 5'-CCC**AAGCTT** **GATATC** **ACTAGT** **ATTTAAAT** **AACGTT** nt 112000G
Hind III *EcoR* V *Spe* I *Swa* I AcI I

CCTGTCGTGTCTGCGCTTTAAGC nt 112023-3'

Reverse 5'-CG**GGATCC** nt 113324TCCAGGCTACACGTGGCCTCGG nt 113303**-3'**
BamH I

Primer pair B:

Forward: GA **AGATCT** nt 114541GGCCGCAGCGCACAGGTCATCG nt 114562-3'
Bgl II

Reverse: CG **GAATTC** **GATATC** **ACTAGT** **ATTTAAAT** **AACGTT** nt 115838G
EcoR I *EcoR* V *Spe* I *Swa* I *Acl* I

AGACGTCCTTAATCGTCCCGAC nt 115860-3'