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DNA immunization with a herpes simplex virus 2 bacterial artificial chromosome

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Abstract

Construction of a herpes simplex virus 2 (HSV-2) bacterial artificial chromosome (BAC) is described. BAC vector sequences were inserted into the thymidine kinase gene of HSV-2 by homologous recombination. DNA from cells infected with the resulting recombinant virus was transformed into E. coli, and colonies containing the HSV-2 BAC (HSV2-BAC) were isolated and analyzed for the expected genotype. HSV2-BAC DNA was infectious when transfected back into mammalian cells and the resulting virus was thymidine kinase negative. When used to immunize mice, the HSV2-BAC DNA elicited a strong HSV-2 specific antibody response that was equal to or greater than live virus immunization. Further, HSV2-BAC immunization was protective when animals were challenged with a lethal dose of virus. The utility of the HSV2-BAC for construction of recombinant virus genomes was demonstrated by elimination of the HSV-2 glycoprotein D (gD) gene. A recombinant HSV-2 BAC with the gD gene deleted was isolated and shown to be incapable of producing infectious virus following transfection unless an HSV gD gene was expressed in a complementing cell line. Immunization of mice with the HSV2 gD-BAC also elicited an HSV-2 specific antibody response and was protective. The results demonstrate the feasibility of DNA immunization with HSV-2 bacterial artificial chromosomes for replicating and nonreplicating candidate HSV-2 vaccines, as well as the utility of BAC technology for construction and maintenance of novel HSV-2 vaccines. The results further suggest that such technology will be a powerful tool for dissecting the immune response to HSV-2.

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Introduction

Several large DNA and RNA viruses have been cloned as bacterial artificial chromosomes (BACs), including poxviruses (Domi and Moss, 2002), herpesviruses [e.g., Epstein-Barr virus, (Delecluse et al., 1998), human and mouse cytomegaloviruses (Borst et al., 1999; Messerle et al., 1997), pseudorabies virus (Smith and Enquist, 2000), herpes simplex virus 1 (Horsburgh et al., 1999b; Saeki et al., 1998; Stavropoulos and Strathdee, 1998)], and coronaviruses [e.g., transmissible gastroenteritis (Almazan et al., 2000)]. A major utility of constructing such recombinant viral BACs is the resulting ease with which the viral genome can be manipulated with prokaryotic genetic techniques to construct recombinant viruses. For example, essential and nonessential virus genes can be rapidly identified without construction of complementing cell lines (Brune et al., 1999). Furthermore, because all manipulations are performed as DNA clones in a prokaryotic system, the chance of isolating a recombinant genome with unrelated mutations should be reduced. Since the mutated genome exists as a DNA clone, sequence analysis of recombinants is facilitated and plaque purification of the resulting recombinant virus is not required.

Viral BACs could also be potentially used for immunization. In theory, a BAC containing the entire genome of a nonreplicating virus could be used to deliver almost the entire spectrum of viral genes as a DNA vaccine. Such an approach using a nonreplicating HSV-1 BAC has recently been described (Suter et al., 1999). However, the future of DNA vaccines is still not clear. While DNA vaccines have shown promise in animal studies, their utility in humans is yet to be proven. Nevertheless, DNA vaccines, particularly

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those expressing numerous antigens to elicit a broad immune response that mimics natural infection, may have a role in new generation vaccines, possibly as part of novel prime-boost combinations using other vectors.

In addition to delivery of nonreplicating viral genomes, BACs could also be used to deliver the genome of a replicating virus for live virus immunization. Most of the advantages (e.g., good immunogenicity and stimulation of humoral and cellular immunity) and disadvantages (e.g., safety concerns and potential virus latency or persistence) of live virus vaccines are well known. Maintenance of live virus vaccines as DNA in the form of a BAC would offer additional advantages to the generation and production of live virus vaccines. A major advantage, as noted above, is that construction of new-generation virus vaccines would be facilitated. In addition, viral vaccine stocks could be re-derived as necessary by transfection of BAC DNA into new qualified cell lines, thus eliminating concerns regarding potential contamination or undocumented virus seed history. Finally, if viral vaccines could be produced and delivered as DNA, concerns about the cell substrates used for vaccine production, including tumorigenicity and the presence of unknown adventitious agents, could be eliminated.

Here, we describe the construction of a herpes simplex virus 2 (HSV-2) BAC and evaluate the ability of this DNA construction to immunize mice and protect them against a lethal challenge of HSV-2. We demonstrate the utility of this HSV-2 BAC system for construction of new genomes by engineering a nonreplicating glycoprotein D (gD)-negative HSV-2. Finally, we show that DNA immunization with the gD-negative BAC DNA also elicits a protective immune response. Thus, BAC technology provides a mechanism to dissect and characterize the contribution of individual viral genes to the generation of protective immunity.

Results

Construction and characterization of a herpes simplex virus 2 bacterial artificial chromosome

To clone the HSV-2 genome as a bacterial artificial chromosome, we constructed a plasmid insertion vector to insert the E. coli F-factor replicon into the viral genome. This insertion plasmid, designated as pSP72tk2-BeloBAC (Fig. 1), contains the 7.5-kb BeloBAC11 sequence which includes the F-factor origin of replication, the choramphenicol acetyltransferase (cat) gene, and the regulatory genes necessary for replication and maintenance at a low copy number in its E. coli host. In addition, the BeloBAC11 sequence is flanked by two approximately 1.75-kb fragments of HSV-2 sequence around the thymidine kinase (tk) gene which are designed to target the BeloBAC sequence to the HSV-2 tk locus for insertion during homologous recombination. Recombinant HSV-2 was generated by co-transfection of pSP72tk2-BeloBAC and HSV-2 genomic DNA into Vero cells, followed by selection for thymidine kinase negative virus in the presence of acyclovir.

Fig. 1. Schematic diagram of the structure of HSV2-BAC. The BeloBAC vector sequences were inserted into the HSV-2 thymidine kinase gene via homologous recombination between HSV-2 and the insertion vector pSP72tkBeloBAC. The single XhoI restriction site in BeloBAC is approximately 2 kb from the HindIII site used for insertion into the tk gene of pSP72tkBeloBAC.
Two independent virus plaques carrying the chloramphenicol gene were identified and re-plaqued one time before preparation of infected cell DNA which was used to transform E. coli DH10B. Chloramphenicol-resistant colonies obtained from each of the two original virus recombinants were selected and analyzed for the presence of BeloBAC and HSV-2 sequences. HSV2-BAC DNA from two independent clones, derived from the two independent virus recombinants, was analyzed by restriction enzyme analysis and Southern blotting to verify the structure of the HSV2-BAC and the insertion of the BeloBAC11 sequences into the thymidine kinase (tk) gene (Fig. 2). The tk gene is located within a 6164-bp XhoI fragment (coordinates 45,174–51,311; GenBank accession number, Z86099) (Fig. 2A, lane 2). Insertion of the 7400-bp BeloBAC sequence (containing a single XhoI site) followed by digest with XhoI results in tk- and BeloBAC-positive fragments of approximately 4300 and 9200 bp (Figs. 2A and B, lanes 1 and 3). DNA from each HSV2-BAC isolate was infectious when transfected into Vero cells; approximately $10^5$ plaque-forming units (pfu) of virus were produced by transfection of 1 μg of HSV2-BAC DNA (data not shown). Virus derived from HSV2-BAC DNA replicated to slightly lower levels than wild-type HSV-2 in Vero cells; HSV2-BAC virus was thymidine kinase deficient as expected, as shown by its ability to replicate in the presence of acyclovir (Fig. 3).

**Immunization with an HSV-2 BAC**

To determine the feasibility of immunization using DNA from the HSV2-BAC, we delivered HSV2-BAC DNA by gene gun and compared the immune response to live virus immunization. Four groups of mice were immunized with either 1 μg of HSV2-BAC DNA, $5 \times 10^4$ pfu of HSV2-BAC virus, $5 \times 10^2$ pfu of HSV2-BAC virus, or 1 μg of control DNA (pBeloBAC). Serum samples were obtained 3 weeks later and analyzed for the presence of HSV-2 specific antibody (Fig. 4A). A single inoculation with 1 g of HSV2-BAC DNA resulted in a readily detectable HSV-2 specific IgG response in all animals, as did inoculation with $5 \times 10^4$ pfu of virus. Only two of five animals inoculated one time with the lower dose of virus responded with detectable HSV-2 specific IgG. Three weeks after immunization, each group of animals was boosted with the same dose of DNA or virus as received in the first immunization; sera were again collected 3 weeks later. After the second immunization with either DNA or virus, all mice in each vaccinated

![Fig. 2. Southern blot analysis of HSV2-BAC.](image)

![Fig. 3. Virus replication analysis of HSV-2 and HSV2-BAC virus.](image)
group sero-converted and an increase in the mean IgG titer in each group was observed. There was a relatively large variability in the specific HSV-2 antibody response in the group receiving the lower dose of HSV2-BAC virus, likely reflecting the poor initial response in three of the five animals. Further analysis of the sera obtained after two immunizations with either HSV2-BAC DNA or 5 \times 10^4 pfu of HSV2-BAC virus revealed high titers of IgG1, IgG2a, and IgG2b in both groups, indicating a broad humoral immune response to both types of immunization (Fig. 4B). HSV-2 specific IgG3 was detectable in all five animals in the HSV2-BAC DNA-immunized group, but in only two of the five animals in the virus-immunized group. In addition, sera obtained after two immunizations with either HSV2-BAC DNA or 5 \times 10^7 pfu of HSV2-BAC virus were capable of neutralizing HSV-2 in vitro [3.06 \pm 0.30 and 2.11 \pm 0.51 (P = 0.007), mean 50% neutralization titers (log 10), respectively]. Finally, mice immunized with HSV2-BAC DNA survived a lethal challenge of HSV-2 (five of five) as did 80% (four of five; P = 1.00) of the animals receiving the higher dose of HSV2-BAC virus (Fig. 5). In the experiment shown in Fig. 5, three of five animals in the group receiving the lower dose and one mouse in the group receiving the higher dose of HSV2-BAC virus succumbed to virus challenge. These animals had the lowest HSV-specific IgG antibody levels after two immunizations in their respective groups (data not shown). The three animals in the low-dose group were the same animals without a detectable HSV antibody after one immunization, suggesting a rough correlation between antibody response and protection against lethal challenge. Taken together, these analyses indicated that gene gun delivery of HSV2-BAC DNA elicited strong HSV-specific antibody responses and substantial protection against lethal challenge, equivalent to or greater than live virus immunization.

Construction of recombinant HSV-2 bacterial artificial chromosomes

One of the major advantages of cloning a large virus such as HSV-2 as a bacterial artificial chromosome is the
ease of constructing other recombinant viral genomes. Such recombinant HSV-2 genomes might find use as newer generation live virus vaccines. We investigated the ease of HSV-2 BAC manipulation by constructing a replication-incompetent genome that was missing the essential glycoprotein D gene. This allowed us to determine the feasibility of BAC immunization with a replication-incompetent HSV-2 virus, and also investigate the immunogenicity of HSV-2 in the absence of one of the major immunodominant viral antigens. For deletion of the HSV-2 gene, we constructed a gene replacement plasmid vector which contained approximately 2 kb of DNA sequences upstream of the gD-coding region linked to approximately 2 kb of sequences immediately downstream of the gD-coding region. The gD sequences target the vector to the gD region of the HSV-2 BAC for deletion of the gD-coding region. In addition, the replacement vector contains a temperature-sensitive origin of replication and marker genes for positive and negative selection during the BAC mutagenesis procedure as described previously (Horsburgh et al., 1999a, 1999b). The absence of the gD gene in the mutagenized HSV-2 BAC was confirmed by PCR analysis (see Materials and methods). The resulting HSV-2 gD-BAC was not infectious when the DNA was transfected into Vero cells (<4 pfu/ml per gram of transfected DNA), but was infectious when transfected into a Vero cell line (VD60) that expresses HSV-1 gD (data not shown). These results verified the genotype and the resulting phenotype of the replication-incompetent HSV-2 gD-BAC.

Immunization with HSV2 gD-BAC

Although missing one of the major immunodominant HSV-2 gD-BAC DNA HSV-2 glycoproteins, it was of obvious interest to determine the ability of HSV2 gD-BAC DNA to elicit an immune response in immunized animals. Mice were immunized two times at 3-week intervals by gene gun inoculation with 1 μg of control DNA (pBeloBAC vector), HSV2-BAC DNA, or HSV2 gD-BAC DNA. HSV-2 specific IgG antibody was detected following each immunization with both HSV2-BAC and HSV2 gD-BAC DNA (Fig. 6), and sera obtained after two immunizations with either HSV2-BAC or HSV2 gD-BAC DNA were capable of neutralizing HSV-2 infectivity in vitro [2.29 ± 0.59 and 1.75 ± 0.25 (P = 0.096), mean 50% neutralization titers (log 10), respectively]. Furthermore, mice immunized with either HSV2-BAC DNA or HSV2 gD-BAC DNA survived a lethal challenge with HSV-2 (Fig. 7).

Discussion

The recent reports that herpesvirus genomes can be cloned and maintained as bacterial artificial chromosomes has facilitated genetic studies of these large DNA virus genomes (reviewed recently in Wagner et al., 2002). The potential applications are numerous and include basic studies of viral pathogenesis, gene identification and characterization, and the development of new viral vectors and vaccines. Effective vaccines are not available for herpes simplex virus 2, the principal causative agent of genital herpes. Furthermore, it is not certain whether a successful vaccine against HSV-2 can be developed, nor is it clear what type of vaccine approach (e.g., live attenuated, subunit, DNA) is most likely to be successful. With these consid-
erations, we reasoned that the advantages of BAC technology could be beneficial for multiple types of HSV-2 vaccine studies, including construction of attenuated virus strains, DNA vaccine evaluation, and the characterization of the contribution of individual viral genes to the generation of protective immunity.

To construct an HSV-2 bacterial artificial chromosome, we followed a procedure similar to that described for construction of an HSV-1 BAC (Horsburgh et al., 1999b). Insertion of the BeloBAC11 sequence into the HSV-2 thymidine kinase gene allowed for selection of the recombinant virus in the presence of acyclovir, followed by isolation of circular DNA that could be recovered by bacterial transformation. This procedure resulted in an HSV-2 with a thymidine kinase negative phenotype. While thymidine kinase is necessary for viral virulence and thus important for viral pathogenesis studies, it is also a target of antiviral drugs such as acyclovir or ganciclovir and thus would be an important safety feature in any live attenuated herpesvirus vaccine. The HSV2-BAC constructed in the present study is not envisioned as a vaccine for HSV-2 per se, but rather is used to demonstrate the feasibility of HSV-2 BAC immunization and the utility of an HSV-2 BAC for further manipulation of the viral genome. Before any HSV vaccine can be effectively evaluated as a vaccine, a thorough characterization of its ability to induce cellular as well as humoral immunity would be necessary, and its protective capabilities in multiple relevant animal models would need to be assessed. In addition, any live HSV-2 virus considered for use as a vaccine would require a demonstrated attenuated phenotype, extensive characterization of the BAC genome including sequence analysis of the genomic termini, reduced or impaired ability to establish and be reactivated from latency, and a functional thymidine kinase gene (for safety reasons). Such targeted manipulations of the viral genome to confer attenuation and impair reactivation from latency could easily be incorporated into a candidate vaccine through BAC mutagenesis for preclinical studies; a thymidine kinase negative BAC virus could be repaired by insertion of an intact thymidine kinase gene at another location in the genome in a relatively straightforward procedure. There have been recent reports that describe alternative sites for insertion of the BAC vector sequences including the intergenic region between the U$_1$3 and U$_1$4 genes of HSV-1 (Tanaka et al., 2003). In addition, methods have been described that allow the removal of BAC vector sequences from herpesvirus BACs (Smith and Enquist, 2000; Tanaka et al., 2003; Wagner et al., 1999). Clearly, the availability of full-length infectious clones of herpesviruses such as HSV-2, and the techniques for their manipulation, provide powerful tools for the development of new generation vaccines.

One of the major goals of the studies reported here was to determine the feasibility of immunization with HSV2-BAC DNA. Two immunizations with 1 μg of HSV2-BAC DNA by gene gun inoculation into mice resulted in a robust HSV-specific IgG antibody response and neutralizing antibody response. We observed some variation in the absolute antibody titer between experiments (e.g., the antibody titer elicited by HSV2-BAC DNA immunization presented in Figs. 4 and 6), which was most likely due to the particular preparation of BAC DNA used for delivery in the experiment. Nevertheless, the HSV-specific antibody response following HSV2-BAC immunization was reproducibly high and the response was consistent in that every animal in five independent experiments responded with detectable antibody (data not shown).

The choice of 1 μg of BAC DNA for delivery by gene gun was arbitrary, but based on considerations of practicality (e.g., relatively small amounts of BAC DNA) and the amount of infectious virus produced from HSV2-BAC DNA. While we did not formally demonstrate that the HSV2-BAC DNA was infectious following in vivo delivery, infectious virus was readily produced following in vitro delivery by transfection, with approximately $10^5$ pfu of virus being produced following transfection of 1 μg of BAC DNA. Since it is difficult to determine the amount of virus produced by in vivo delivery of HSV2-BAC DNA and it is not clear that in vivo delivery of DNA is as efficient as in vitro transfection, we compared in vivo delivery of 1 μg of BAC DNA to two concentrations of HSV2-BAC virus, $5 \times 10^4$ and $5 \times 10^2$ pfu. High levels of HSV-specific IgG antibody responses were elicited by both HSV2-BAC DNA immunization and the higher dose of virus inoculation ($5.15$ and $4.07$ ($P = 0.016$), respectively). Similarly, good neutralizing antibody responses were produced after immunization, and the antibody isotype responses elicited in each group were qualitatively similar. Some caution should be observed in evaluation of the significance of the antibody titers and neutralization titers between the DNA- and the virus-infected animals. Clearly, there is a dose response effect that is dependent on the amount of live virus given for immunization (Fig. 4A), and it is very likely that a further increase in the dose of virus would have produced even higher levels of specific antibody. We also have not attempted a dose-ranging study with the HSV2-BAC DNA. Most importantly, the results indicated that HSV2-BAC DNA was strongly immunogenic and suggested the feasibility of virus immunization by DNA delivery.

To assess the feasibility of DNA immunization with a replication-defective HSV-2 BAC, the HSV-2 gD gene was eliminated by mutagenesis of the HSV2-BAC. The gD gene was chosen for elimination to validate the power of the BAC mutagenesis procedure to construct nonreplicating virus mutants. Further, deletion of an immunodominant antigen such as gD would provide a rigorous test of the potential immunogenicity of BAC DNA. Elimination of the essential HSV-2 gD gene was relatively straightforward and did not require prior construction of a complementing cell line. However, a Vero cell line expressing the HSV-1 gD was utilized to verify the gD-negative phenotype and to produce
gD-negative virus stocks. Because of the difficulty in constructing cell lines expressing sufficient levels of multiple complementing genes, BAC mutagenesis procedures are therefore especially useful for mutagenesis and isolation of viral genomes containing multiple disabling mutations.

DNA immunization with a replication-defective HSV-1 BAC has been reported previously (Suter et al., 1999). In that study, a packaging-defective HSV-1 genome cloned as a BAC (fHSV pac) (Saeki et al., 1998) induced a broad immune response that protected immunized mice from intracerebral challenge. An HSV-1 antibody response was detected after one gene gun immunization of BAC DNA, and increased after a second and third immunization; neutralizing antibody was detected after two immunizations. Like our HSV2 gD-BAC, the fHSV pac should replicate and produce noninfectious virus-like particles. The fHSV pac on the other hand should express the entire complement of HSV-1 genes including HSV-1 gD. It is somewhat difficult to compare the relative protective capacities and immune responses elicited by the packaging defective HSV-1 BAC and the replication-competent and -incompetent HSV-2 BACs described in our study. In the HSV-1 study, BAC immunization is compared to an HSV-1 gD-deletion virus. In our HSV-2 study, we have compared HSV-2 BAC DNA immunization to replicating virus generated from the BAC clone and to an HSV-2 gD-deletion BAC. Nevertheless, the fact that fundamentally different HSV BACs can elicit protective immune responses in at least some animal models strongly suggests the feasibility of BAC DNA immunization.

While gene gun inoculation of animals with HSV2 gD-BAC DNA elicited an HSV-specific antibody and neutralizing antibody response that was lower than the corresponding HSV2-BAC DNA response, animals were still protected against a lethal virus challenge. These results were not completely surprising. Immunization with HSV, purified gD glycoprotein, or gD DNA produces a strong humoral response to gD and is protective. Nonetheless, the immune response to HSV is complex and directed to many viral antigens in addition to gD. Many other HSV glycoproteins elicit an antibody response that is protective in various animal challenge models. More importantly, the ability to systematically and sequentially eliminate individual and multiple HSV-2 antigens using BAC technology provides a powerful tool for dissecting the immune response to HSV-2.

The advantages of cloning large viral genomes as BACs are numerous. Perhaps the most exciting is the ability to construct recombinant genomes that would be difficult under other circumstances, as described above and previously for other herpesviruses (Brune et al., 2000). The possibility that viral vaccines could be delivered as BAC DNA opens up other interesting possibilities that could facilitate vaccine development. If delivery of live virus via BAC DNA proved to be practical, technical problems associated with poor growth and yields of mutated virus even on a complementing cell line could be circumvented. Of course, issues associated with the large-scale production of BAC DNA would have to be addressed. Further, there are obvious advantages conferred by maintenance of novel viral vaccine stocks as BAC DNA and bacterial stocks containing the recombinant BAC. In such cases, new vaccine stocks could be re-derived as required to address emerging concerns regarding the virus seed history or the material used for virus stock preparation. The ability to rapidly construct novel HSV-2 genomes using BAC technology as described here should facilitate the development and evaluation of candidate HSV-2 vaccine candidates.

Materials and methods

Cells and viruses

Vero cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax-1, and 0.05 mg/ml Gentamicin (Gibco-BRL, Grand Island, NY). VD60 cells, a Vero cell line that expresses the HSV-1 gD (Ligas and Johnson, 1988), were a gift from Dr. David C. Johnson, Department of Molecular Microbiology and Immunology, Oregon Health Sciences University. These cells were maintained as suggested in Eagle MEM lacking histidine (MEM—his) supplemented with 1 mM histidinol (Sigma Chemical Co., St. Louis) and 5% FBS. Before infection, VD60 cells were passaged two times in DMEM containing 10% FBS. The strain MS of herpes simplex virus 2, originally isolated from the brain of a patient with multiple sclerosis, was obtained from the ATCC, plaque-purified, propagated, and titrated in Vero cells. Viral DNA was isolated as described elsewhere (Denniston et al., 1981).

Construction of plasmids

To construct the insertion plasmid, pSP72tk2-BeloBAC, we first cloned two regions of the HSV-2 thymidine kinase gene into the cloning vector pSP72 (Promega Corporation, Madison, WI). The two tk fragments, flank-1 and flank-2, were made by PCR amplification from HSV-2 genomic DNA template. Primer sequences were designed based on the published data for HSV-2 strain HG52 (Dolan et al., 1998). Flank-1 consisted of the 3’most 585 bp of the tk coding sequence plus an additional 1.167 kb of downstream sequence and was amplified by the primers, tk-a (5’ATCTCGAGCCCCCATGAAAGCCTTCCCG; coordinates 45706–45732) and tk-b (5’GGAACCTTGACCCC CCAGGCGTGTGTT; coordinates 47460–47434), incorporating an XhoI and a HindIII site (in bold), respectively, at the 5’end of each primer. The sequence of flank-2 consisted of the 5’547 bp of the tk coding sequence, plus an additional 1.176 kb of upstream flanking sequence. Flank-2 was
amplified with the primers tk-c (5′GTCAAGCTTCCCAT GAGGTACCGCGCG; coordinates 47450–47476) and tk-d (5′GTCTAGAGGGGATGGTGACGCGGCA; coordinates 49176–49149), incorporating a HindIII and a XbaI (in bold) site, respectively, at the 5′end of the primers. This cloning strategy resulted in the intermediate insertion plasmid, pSP72-tk2, which contains approximately 3.47 kb of the tk gene locus and flanking HSV-2 sequences, with a unique HindIII restriction site near the middle of the tk coding region. The HindIII restriction site at this position allowed easy insertion of the linearized 7.5-kb BeloBAC11 vector (Research Genetics, Huntsville, AL) to generate pSP72tk2-BeloBAC. The authenticity of all constructs was confirmed by sequencing in an ABI-377 DNA sequencer (Applied Biosystems, Foster City, CA). PCR products were initially cloned using the Zero Blunt Topo PCR cloning kit (Invitrogen, Carlsbad, CA). Routine isolation and purification of plasmids was performed using Qiagen plasmid purification kits and protocols described by the manufacturers (Qiagen Inc., Valencia, CA).

To construct the glycoprotein D (gD)-deletion replacement vector, we amplified two regions of the HSV-2 gD gene by PCR and cloned these fragments into the cloning vector pGEM3 (Promega). One fragment consisted of the 2016 bp immediately upstream of the first ATG of the gD-coding sequence; the second fragment consisted of the 2106 bp immediately downstream from the end of the gD-coding sequence. This approximately 4-kb region with gD-coding sequences deleted was transferred to the Gateway entry vector pENTR1A (Invitrogen). The resulting plasmid construction, pENTR-1A-gD5678, was used to transfer the gD region into a BAC gene replacement vector, pKO5.2-C.1, to yield pKO5.2-C.1-gD5678 for BAC mutagenesis. The vector pKO5.2-C.1 is a derivative of the previously described gene replacement vectors pK05 and pKO5.1 (Horsburgh et al., 1999a, 1999b), modified for use as a Gateway destination vector by the insertion of a gene cassette containing attR recombination sites into the multiple cloning region (manuscript in preparation).

**BAC mutagenesis**

Mutagenesis of the HSV2-BAC was essentially as described previously (Horsburgh et al., 1999a, 1999b). Briefly, RR1 electrocompetent cells were transformed with HSV2-BAC DNA and selected for chloramphenicol resistance. The electroporation conditions employed 0.1-cm cuvets (BTX, San Diego, CA) and a 1.8-kV setting on a Transporator Plus (BTX). Clones were confirmed by PCR with three independent HSV-2 specific primer pairs and a primer pair amplifying a fragment in the chloramphenicol acetyltransferase (cat) gene. Electrocompetent RR1 cells harboring the HSV-2 BAC were prepared as previously described (Ausubel et al., 1987). Electrocompetent RR1 cells containing the HSV2-BAC were electroporated with 10 ng of pKO5.2-C.1-gD5678 DNA, and plated onto chloramphenicol/zeocin (Cam/Zeo) plates in serial dilutions at 30 °C. The following day, multiple colonies were picked into 1 ml of LB broth and plated in serial dilutions onto Cam/Zeo plates at 43 °C. Colonies at 43 °C were analyzed by picking several into 1 ml LB each and immediately plating out 10 and 100 μl onto sucrose/chloramphenicol (Suc/Cam) plates at 30 °C. Approximately 10 colonies from each plate, representing an original 43 °C colony, were transferred to grids on Suc/Zeo and Cam LB plates at 30 °C. Colonies which grew on Cam plates but not on Zéo/Suc, (i.e., Cam+, Zéo/Suc−) were picked into 100 μl of 10 mM Tris pH 8.0 for colony PCR using HSV-2 specific primers along with a primer pair flanking the gD-coding region. Positive colonies were streaked out onto Cam plates and grown at 37 °C. Clones were grown in Cam-LB media for glycerol stocks and subsequent large preparations of HSV2 gD-BAC DNA using the Large Construct kit (Qiagen Inc.).

**Animals and immunization**

BALB/cByJ mice were purchased from Jackson Laboratories, Bar Harbor, ME, and housed in cages at a core facility in the Center for Biologics Evaluation and Research (CBER), Bethesda, MD. Sterile food and drinking water were supplied ad libitum, and general care and handling procedures were performed according to a protocol approved by the Animal Care and Use Committee of CBER/FDA. Mice were used in experiments at between 8 and 12 weeks of age, and age-matched within an experiment. DNA immunizations by gene gun inoculation and HSV-2 intraperitoneal challenges were performed as previously described (Meseda et al., 2002). Live virus immunizations were by intradermal injection at the base of the tail.

**Antibody and neutralizing antibody determination**

Detection of antibody to HSV glycoprotein by ELISA (Pachl et al., 1987) was performed as previously described (Meseda et al., 2002; Nass et al., 1998), using a mixture of HSV-2 glycoproteins or an inactivated HSV-2-infected cell extract. Endpoint titer was defined as the highest dilution of serum that gave an A405 value that was 2-fold greater than that of the matched dilution of normal prebleed mouse serum and was also greater than 0.050. Neutralizing antibody assays were performed using 96-well plates containing 5 × 10⁴ Vero cells and 5 × 10³ pfu of HSV-2 per well as previously described (Meseda et al., 2002; Nass et al., 1998). Sera were assayed from individual mice within each group tested (n = 5).

**Statistical analysis**

 Significant differences in antibody titers between groups, defined as P < 0.05, were analyzed using an unpaired, two-
tailed Student’s *t* test with InStat software. Significant differences in survival between challenged groups of mice were analyzed using Fisher’s exact test with InStat software.

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