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Review Article

Herpesvirus BACs: Past, Present, and Future

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The herpesviridae are a large family of DNA viruses with large and complicated genomes. Genetic manipulation and the generation of recombinant viruses have been extremely difficult. However, herpesvirus bacterial artificial chromosomes (BACs) that were developed approximately 10 years ago have become useful and powerful genetic tools for generating recombinant viruses to study the biology and pathogenesis of herpesviruses. For example, BAC-directed deletion mutants are commonly used to determine the function and essentiality of viral genes. In this paper, we discuss the creation of herpesvirus BACs, functional analyses of herpesvirus mutants, and future applications for studies of herpesviruses. We describe commonly used methods to create and mutate herpesvirus BACs (such as site-directed mutagenesis and transposon mutagenesis). We also evaluate the potential future uses of viral BACs, including vaccine development and gene therapy.

1. Introduction

Human herpesviruses are a leading cause of human viral disease, second only to influenza and cold viruses [1]. Herpesviruses contain a large double-stranded DNA genome that ranges in size from 125 to 240 kilobase pairs. Their genomes are tightly packed in virions in a linear form but become circularized once they enter the nucleus (where they replicate) [2]. This circularization becomes important for herpesvirus BAC construction, which we will discuss in this paper. All herpesviruses undergo a latent infection following primary infection. During latency, the virus remains dormant and is able to evade the host immune system. Under several circumstances, lytic replication can be reactivated in latent viruses, thereby causing various types of disease. This characteristic makes herpesvirus infections especially hard to treat.

There are eight human herpesviruses: herpes simplex virus 1 (HSV-1 or HHV-1), herpes simplex virus 2 (HSV-2 or HHV-2), varicella-zoster virus (VZV or HHV-3), Epstein-Barr virus (EBV or HHV-4), human cytomegalovirus (HCMV or HHV-5), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) [2]. HSV-1 and 2 are the causal agents of oral and genital herpes, respectively [3]. VZV is the causal agent of chickenpox and shingles [4]. HCMV is a major cause of infectious morbidity and mortality in immunocompromised individuals and developing fetuses, and HCMV-caused disease is called cytomegalovirus inclusion disease (CID) [5]. HHV-6 is associated with roseola [6]. EBV is associated with a number diseases, most notably infectious mononucleosis (colloquially known as *mono* or *kissing disease*) and Burkitt's lymphoma [7]. As the name implies, KSHV is associated with Kaposi's sarcoma [8], but KSHV can also cause B-cell lymphoma.

Ever since the creation of the first herpesvirus bacterial artificial chromosome (BAC) mutant (for murine cytomegalovirus, MCMV) over a decade ago, mutagenesis

using BAC technology has been proven to be an invaluable tool for studying herpesvirus pathogenesis [9–13]. BACs are especially useful for studying herpesviruses because the DNAs of these viruses are too large to be cloned in individual plasmids or cosmids [13]. Although Yeast Artificial Chromosomes (YACs) can also be used to carry large genomic sequences, BACs are more suitable for viral studies because BACs are more stable than YACs and are less prone to undesired genomic rearrangements and contamination with yeast DNA [14, 15]. BACs are especially useful for many herpesviruses because of their especially slow replication rate and the relative ease and accuracy of producing BAC mutants. Additionally, viral BACs in *E. coli* can be more stable than traditional or natural viral mutants. In particular, viral BAC-containing *E. coli* strains can be stored at −80°C, and new viruses can quickly be produced from BAC DNAs. In contrast, clinical isolates can become attenuated by repeated culture passage *in vitro*. However, the stability of viral BACs needs to be periodically checked, especially because viruses will be subject to different selective pressures in bacterial and mammalian cells, and viral BACs may acquire undesired mutants after replicating in *E. coli* [9, 10]. For example, repetitive sequences in the viral DNA have been shown cause unwanted phenotypic changes when viral BACs are replicated in *E. coli* [16]. Researchers also need to be able to produce a variety of different type of BAC mutants (such as stop-codon mutants or premature-frameshift mutants) in order to ensure that a mutation is due to a change in the functionality of a given gene (as opposed to disrupting cis-regulatory elements or altering the positioning of enhancers).

Viral BACs are created by inserting a BAC vector sequence into a viral genome. Methods for the construction of viral BACs are outlined in the second section of this paper. There are two commonly used methods to mutagenize BAC DNA: random transposon and site-directed mutagenesis. Site-directed mutagenesis utilizes homologous recombination to create specific mutations in viral genes. In contrast, transposon mutagenesis creates a large diversity of BAC mutants, but mutagenesis is random, and sequencing or PCR is required to determine the mutation site. A detailed description of the methods for the mutagenesis of viral BACs is provided in the third section of this paper.

Viral BACs have been created for every human herpesvirus except HHV-7 and several animal herpesviruses that are frequently used as animal models for studying viral pathogenesis (including murine cytomegalovirus, pseudorabies virus, and herpesvirus saimiri). We will review some specific studies of BAC creation and mutagens for both human and nonhuman herpesviruses in the fourth section of this paper. We will also discuss global mutagenesis studies in the fifth section of this paper. A list of human herpesvirus BACs is provided in Table 1, and a list of animal herpesviruses is provided in Table 2. There are also a number of other useful applications for BAC mutants (such as vaccine development and gene therapy), and we will review potential future uses of viral BAC mutants in the concluding section of this paper.

2. Methods for BAC Construction

2.1. Features of the BAC Vector. The crucial feature that defines a viral BAC is the presence of a BAC vector within the viral genome [15]. A typical BAC vector is about 10 kilobase pairs long and must have an origin of replication (*e.g., oriS*), genes necessary for BAC replication (such as *repE*), and genes to control the rate of replication in order to limit the copy number to one or two BACs per bacterial cell (such as *parA* and *parB*). An antibiotic resistance marker (such as chloramphenicol) must also be contained within the BAC vector in order to select only the bacterial colonies containing the BAC herpesvirus of interest. The BAC vector must also be flanked by 500–1000 base pairs that are homologous to the target sequence where the BAC vector will be inserted. In order to isolate BAC-containing recombinant virus, a BAC vector should also carry a selectable marker (such as GFP, beta-galactosidase, antibiotic resistance genes, or metabolic genes). In addition, two loxP sites are often included at both ends of the BAC sequence so that the BAC vector can be excised out when recombinant viruses are generated (see discussion below).

2.2. Direct Insertion of BAC Vector into Viral Genome via Homologous Recombination. One commonly used method of constructing herpesvirus BACs involves inserting a BAC vector into a specific site of the viral genome via homologous recombination [12, 17, 21, 24–26, 31, 32]. For this process, the BAC vector with flanked viral genomic sequences is linearized using restriction enzymes and cotransfected with purified viral genomic DNA into viral permissive cells. Some herpesviruses, such as HCMV and MCMV, contain large genomes and limited capsid capacity. Insertion of a 10-Kb BAC vector will cause severe growth defects in these viruses [50]. Therefore, replacement of a long nonessential region of the viral genome is required for viral BAC construction. If necessary, the original viral sequence can be reinserted once the BAC [51] or moved to a different location in the viral genome [23]. Homologous recombination takes place in the cells, and a recombinant virus carrying a BAC vector will be produced. Viral plaques are purified based upon the presence of a trait defined by a selectable marker. For example, recombinant virus infection will show green fluorescent plaques if the BAC vector contains a GFP expression cassette as a selectable marker. The BAC vector-containing viral DNA is isolated from infected cells and again inserted into a RecA *E. coli* strain, such as DH10B, via electroporation. Although herpesvirus DNA is large and difficult to transform into *E. coli*, the fact that herpesvirus genome circularizes during replication makes this step feasible. Bacterial cells containing viral BACs can be selected based upon the antibiotic marker present in the BAC vector (e.g., chloramphenicol, as described above). If a viral BAC can be stably maintained and replicated in *E. coli*, drug-resistant colonies will be obtained. Site-directed mutagenesis can also occur during this step (see the following section). The viral BAC DNA is purified from *E. coli* and restriction enzyme digestion and, sometimes, partial sequencing analyses are performed to confirm that there have been no major mutations (deletions)

Table 1: BAC-based human herpesvirus studies.

Virus	Global deletion?	Representative studies
Herpes Simplex Virus-1 (HSV-1)	No	Horsburgh et al. 1999 [17] Saeki et al. 1998 [18] Stavropoulos and Strathdee 1998 [19]
Herpes Simplex Virus-2 (HSV-2)	No	Meseda et al. 2004 [20]
Varicella Zoster Virus (VZV)	Yes	Nagaike et al. 2004 [21] Zhang et al. 2007 [22] Wussow et al. 2009 [23]
Epstein-Barr Virus (EBV)	No	Delecluse et al. 1998 [24]
Cytomegalovirus (CMV)	Yes	Borst et al. 1999 [25] Marchini et al. 2001 [26] Hahn et al. 2002 [27] Murphy et al. 2003 [28] Sinzger et al., 2008 [29] Dulal et al. 2009 [30]
Human Herpesvirus 6 (HHV-6)	No	Borenstein and Frenkel 2009 [31]
Human Herpesvirus 7 (HHV-7)	No BAC	NA
Kaposi's Sarcoma-Associated Herpesvirus (KSHV)	No	Delecluse et al. 2001 [32] Zhou et al. 2002 [33]

In most cases, "representative studies" represent the first BAC produced for each human herpesvirus. Hahn et al. 2002, Marchini et al. 2001, and Murphy et al. 2002 correspond to creation of BACs for other alternative strains of HCMV. Zhang et al. 2007 and Dulal et al. 2009 refer to the first BACs with a luciferase reporter genes.

Table 2: Selected BAC-based animal herpesvirus studies.

Virus	Global deletion?	Representative studies
Bovine Herpesvirus Type 1 (BHV-1)	Yes	Mahony et al. 2002 [34] Robinson et al. 2008 [35]
Equine Herpesvirus Type 1 (EHV-1)	No	Rudolph et al. 2002 [36]
Feline Herpesvirus Type 1 (FHV-1)	No	Costes et al. 2006 [37]
Guinea Pig Cytomegalovirus (GPCMV)	No	McGregor and Schleiss 2001 [38]
Herpesvirus Saimiri (HVS)	No	White et al. 2003 [39]
Koi Herpesvirus (KHV)	No	Costes et al. 2008 [40]
Marek's Disease Virus (MDV)	No	Schumacher et al. 2000 [41]
Murine Cytomegalovirus (mCMV)	Yes	Messerle et al. 1997 [12] Brune et al. 1999 [42] Bubeck et al. 2004 [43]
Murine Gammaherpesvirus 68 (MHV-68)	Yes	Adler et al. 2000 [44] Song et al. 2005 [45]
Pseudorabies Virus (PrV)	No	Smith and Enquist 1999 [46]
Rhesus Cytomegalovirus (rhCMV)	No	Chang and Barry 2003 [47]
Rhesus Rhadinovirus (RRV)	No	Estep et al. 2007 [48]
Turkey Herpesvirus (HVT)	No	Baigent et al. 2006 [49]

In most cases, important BACs represent the first BAC produced for a given virus. The work of Brune et al. (1999), Bubeck et al. (2004), Robinson et al. (2008), and Song et al. (2005) are global mutagenesis studies.

in the BAC DNA [22]. The integrity of the BAC can also be confirmed via next-generation sequencing, and this practice may become commonplace in the future. Once the integrity of the viral BAC is confirmed, large amounts of the BAC DNA can be produced in bacterial cells before transfection into mammalian cells. Since the large herpesvirus genome is difficult to transfect, the ability to produce large amounts and high quality of viral BAC DNA has been a very useful development that has significantly aided the study of herpesvirus pathogenesis. The process is outlined in the context of the production of the HCMV BAC in Figure 1.

2.3. Using Overlapping Cosmids to Produce Viral BACs. Herpesvirus BACs can also be constructed using a set of overlapping cosmids covering the whole genome of a virus [18, 19, 22]. This method was necessary for the creation of the VZV BAC because it is difficult to isolate the full-length VZV genome and purify recombinant viral plaques due to the highly cell-associated nature of the virus [21]. For this process, multiple cosmids (usually 3–6) containing partial herpesvirus genomic sequences are constructed such that each cosmid will contain an overlapping sequence when linearized, and this allows the cosmids to recombine into one large virus genome via homologous recombination in a eukaryotic cell. Recombinant viruses could be produced using overlapping cosmids prior to the creation of viral BACs [54–58]. Insertion of a BAC vector into one of the genomic cosmids via homologous recombination is the key step that allows the rapid creation of recombinant viruses that can be genetically manipulated in *E. coli*. BAC vectors can also be inserted into a genomic sequence using the restriction enzyme digestion of viral DNA along with BAC vector, followed by ligation and the Hirt extraction of the recombinant virus [31]. After insertion of the BAC vector, the overlapping cosmids are combined and the resulting viral BAC DNA is circularized following transfection into mammalian cells. Plaques containing the BAC vector can be visualized using a selectable marker, such as GFP. At this point, the process of viral BAC production is identical to the method of direct insertion (see above). This process is outlined in the context of the production of the VZV BAC in Figure 2.

2.4. BAC Visualization Markers. By adding a number of features to the viral BAC genome, it is possible to visualize and quantify viral replication and thereby study viral pathogenesis. For example, a GFP reporter gene is often inserted into BAC DNAs in order to visualize *in vitro* infections in cell culture [18, 21, 24, 32]. Viral GFP is expressed using SV40 promoter and polyadenylation signals, and this allows the GFP gene to be expressed during the appropriate stages of viral replication [26]. A luciferase reporter can also be inserted into viral BACs, which insertion is especially useful for studying *in vivo* infections in organ cultures and in SCID-hu implants [22, 30, 53, 59]. Luciferase activity correlates with viral replication, and the luciferease reporter also allows for multiple measurements within the same culture or host (which is much more efficient than titration assays, which would require mice to be sacrificed for each measurement). In addition to aiding in the study of viral pathogenesis, EBV and HSV viral vectors with luciferase can also be useful for developing gene therapy treatments [60, 61] (for more about gene therapy, see the final section of this paper). Prior to the invention of herpesvirus BACs, it was more difficult to utilize luciferase to study viral replication. For example, one study utilized a human cell line with a luciferase reporter gene with an HSV promoter such that the expression of luciferase correlated with HSV infection [62].

Integrity of visualization markers can be checked periodically using the *in vitro* infection of human cells in order to ensure that undesired, accumulated mutations have not compromised the integrity of the luciferase reporter gene. If serial dilutions of viral plaques reveal that visualization markers are consistently present in the vast majority of plaques, then it should be safe to assume that reporter gene expression will accurately correlate with viral replication [22]. Viral BAC integrity can also be periodically confirmed by isolating DNA and performing restriction digest analysis as well as the random sequencing of gene sequences.

Once the viral BAC is created, functional analysis of each viral gene can be conducted by site-directed mutagenesis of the viral BAC DNA. The two most commonly used methods for BAC DNA manipulation are site-directed mutagenesis and transposon mutagenesis.

3. Methods for BAC Mutagenesis

3.1. Use of E. coli for Site-Directed Mutagenesis via Homologous Recombination. Genetically modified *E. coli* (most commonly, DY380-derived strains) have become a very popular tool to help create recombinant DNA using homologous recombination [63, 64]. Wild-type *E. coli* is ineffective at inducing homologous recombination in foreign DNA because linear DNA is commonly degraded by RecBCD exonuclease (*exo*). In order to circumvent this problem, DY380-derived strains contain a temperature-sensitive λ phage containing one gene to temporarily repress RecBCD (*gam*), as well as two genes (*exo* and *beta*) utilized for homologous recombination via double-strand break repair [64]. More specifically, exonuclease degrades DNA from the 5′ end of double-strand break sites, while Beta binds to and protects the 3′ from further degradation [65, 66]. These overhangs from double-strand breaks allow recombination between viral and plasmid DNA. Because the λ phage is temperature sensitive (due to the expression of a temperature-sensitive λ cI-repressor), linear DNA uptake and recombination can occur within a few minutes when the cell-culture temperature is increased from 32 to 42°C [64]. This allows the bacterial cells to function normally when grown at 32°C, which is necessary for BAC production

E. coli also requires thousands of homologous base pairs in order for recombination to occur. Addition of the modified temperature-sensitive λ phage is important because this allows homologous recombination to occur within a relatively small region of the homologous sequence, which is important because BAC mutants are usually created using PCR-amplified gene sequences with about 40 base pairs of flanking sequences that are homologous to the viral BAC [63].

There are also alternatives to the *exo-gam-beta* system for homologous recombination. For example, plasmid-based systems can also be used to allow for creation of recombinant DNA via homologous recombination in *E. coli* [67]. There are also tools for "markerless" genetic manipulation, including technologies that allow for site-directed mutagenesis without the use of a selectable marker [68].

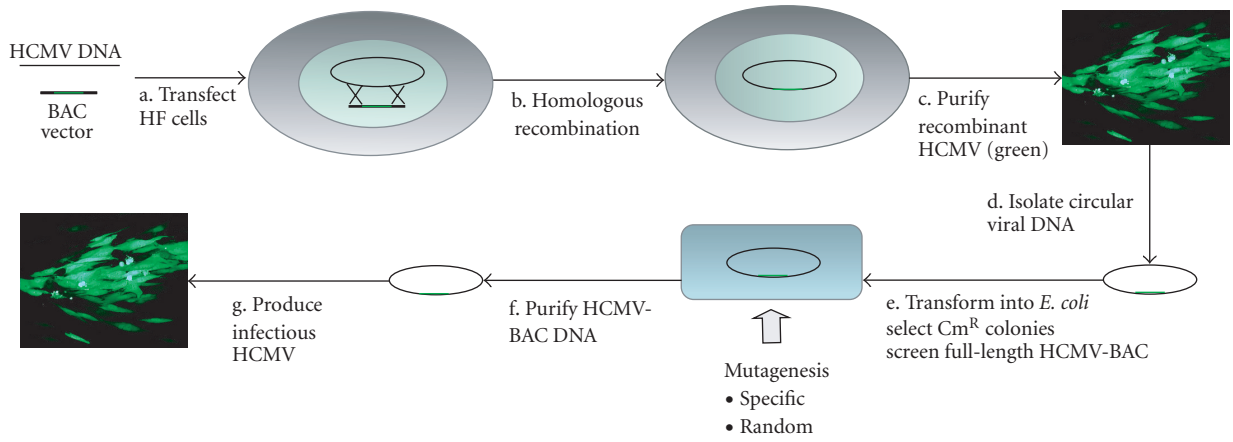

Figure 1: Construction of $HCMV_{BAC}$. (a) HCMV genome and bacterial plasmid with BAC vector that has been linearized and transfected into human fibroblast cells. (b) BAC vector is inserted into HCMV genome by homologous recombination. The viral genome (now containing the BAC vector) will naturally circularize during replication. (c) HCMV viral BACs are selected based upon expression of a GFP cassette within the BAC vector. (d) Fluorescent plaques are isolated, and viral BAC DNA is extracted. (e) Viral BACs inserted into *E. coli* cells via electroporation. Successful integration of the BAC vector into the viral genome can be confirmed by (1) selecting colonies with antibiotic resistance resulting in the chloramphenicol-selectable marker in the BAC vector and (2) confirming the BAC genome sequence has not gained any undesired mutations by using restriction digest analysis to compare the BAC DNA to the original viral DNA. (f) If desired, mutagenize the HCMV viral BAC (via site-directed mutagenesis or random transposon mutagenesis). Either way, viral BAC DNA must be isolated via Maxiprep for transfection back into human cells. (g) Transfect BAC DNA into human fibroblast cells in order to produce infectious virus.

Table 3: Global studies of human herpesvirus gene function.

Study	Virus	No. of essential genes	No. of augmenting genes	No. of nonessential genes	No. of repressive genes
Dunn et al. 2003 [52]	HCMV (Towne)	45	35	68	4
Yu et al. 2003 [50]	HCMV (AD169)	41	27	88	0
Zhang et al. 2010 [53]	VZV	44	8	18	0

Phenotypes are considered for growth in human fibroblast cells for HCMV and human melanoma (MeWo) cells for VZV.

3.2. Site-Directed Mutagenesis Using a Positive Selectable Marker. One popular method to manipulate herpesvirus BACs is to use site-directed mutagenesis to insert foreign elements into, delete DNA fragment from, or make point mutation in the BAC genome [9, 10, 13, 69]. There are multiple ways to carry out site-directed mutagenesis of a viral BAC. For example, selectable markers are necessary to confirm the successful mutation of a viral BAC, and selectable markers can take the form of a positive selectable marker (such as an antibiotic resistance gene) or negative selectable marker (such as a metabolic gene). The well-established method of producing BAC mutants using an antibiotic resistance gene as a positive selectable marker is described in this paragraph and outlined in Figure 3(a). Basically, the antibiotic marker is inserted at a region of interest in the viral BAC using homologous recombination. Only bacterial cells containing the desired mutation will be able to grow in the presence of antibiotic. Selectable markers will need to be carefully selected; for example, the mutation site cannot contain the same antibiotic marker as the BAC vector. A detailed overview of this method is shown in Figure 3(a). The primary advantage to this system is that it allows for precise modification of the BAC viral genome. Only deletion mutants can be produced using a single round of positive selection, and these deletion mutants have the potential to significantly alter viral genomic structure and affect regulation of nearby genes. Therefore, the integrity of the viral genome must be confirmed by creating a rescue virus. Thus, the primary disadvantage to this system is that it requires two rounds of selection and is very time consuming.

In order to confirm that mutants produced via homologous recombination with an antibiotic marker show a phenotype that can be directly attributed to the designed mutation, a rescue virus containing the original genomic sequence musts be created. This is typically achieved with via mutagenesis with a second positive selectable marker (although the newly developed "Gene Capture" method [30] can also be used when the genomic region of interest is too large to be amplified via PCR). A detailed description of method for producing a rescue virus is provided in

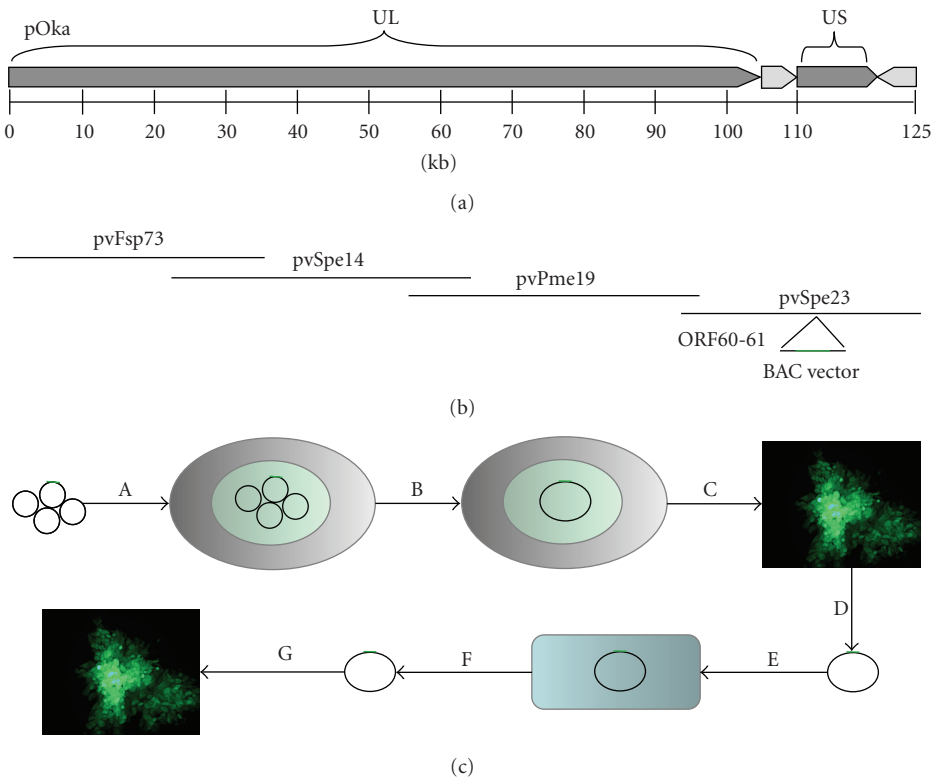

FIGURE 2: Construction of VZV_{BAC}. (a) Schematic diagram of a VZV pOka genome. The 125-kb genome, VZV, contains a unique long (UL) and a unique short (US) segment. (b) Four cosmids containing overlapping VZV genomic segments are shown. A BAC vector was inserted between ORF60-61 in a VZV cosmid, pvSpe23, by homologous recombination. The BAC vector carries a GFP and a CM^R marker. (c) VZV_{BAC} construction. (A) the BAC-containing cosmid was cotransfected with the three complementary cosmids into MeWo cells; (B) homologous recombination between these four cosmids forms a circular full-length VZV genome; (C) the recombinant virus replicated and produced a green plaque; (D) the circular DNA was isolated from infected cells and (E) transformed into *E. coli* and selected for CMR colonies; (F) the VZV_{BAC} DNA was isolated from *E. coli* and verified by restriction digestion and partial sequencing; (G) infectivity and integrity of the VZV_{BAC} were tested by transfecting BAC DNA into MeWo cells and producing VZV virus.

Figure 3(b). Production of a rescue virus requires another round of positive selection using a different marker. This time, the selectable marker sequence must be flanked by sequences that will allow later excision from the genome (e.g., sequences flanked by loxP sequences can be removed using Cre). This method of BAC excision will work for any foreign element, including the BAC vector. For this reason, BAC sequence excision methods should be carefully considered, depending upon the design of the BAC. For example, it will become problematic if both the BAC vector and gene-specific antibiotic marker contain the same flanking excision sequences (such as loxP) because this is likely to cause the removal of a much larger sequence of genomic DNA than originally intended. If the viral BAC has not accumulated any undesired mutations, then the rescue virus should behave exactly like a wild-type viral BAC. As described in the final section of this paper, mutagenesis methods utilizing two rounds of positive selection can also be applied to produce certain types of mutants (such as frame-shift or premature stop-codon mutants) as well as gene fusions (such as GFP-fusion or epitope-tagged genes) [70–72].

3.3. Site-Directed Mutagenesis Using a Negative Selectable Marker. Site-directed mutagenesis can be accomplished using *galK* as a negative selectable marker. The disadvantage of using antibiotic resistance genes as a selectable marker (as described above) is that the flanking homologous sequences that contain LoxP or FRT might cause the loss of viral DNA during the process of making viruses in mammalian cells. Thus, the process of making rescue mutants using an antibiotic marker is complex and can induce unintended mutations. However, these problem can be avoided by using a negative selectable marker (such as *galK*) [69]. The process of this method is outlined in Figure 3(c). Briefly, viral BAC DNA is inserted into *E. coli* SW102 by electroporation. Unlike DY380 *E. coli* cells (which lack a functional galactose operon), SW102 has a galactose operon from which only galK has been deleted. Thus, SW102 with a copy of a functional galK inserted can grow when the only provided carbon source is galactose. Therefore, gene modifications can be made by amplifying galK using PCR primers homologous to the BAC sequence at the genomic locus of interest and then selecting bacterial colonies that can grow in minimal medium with only the addition of galactose.

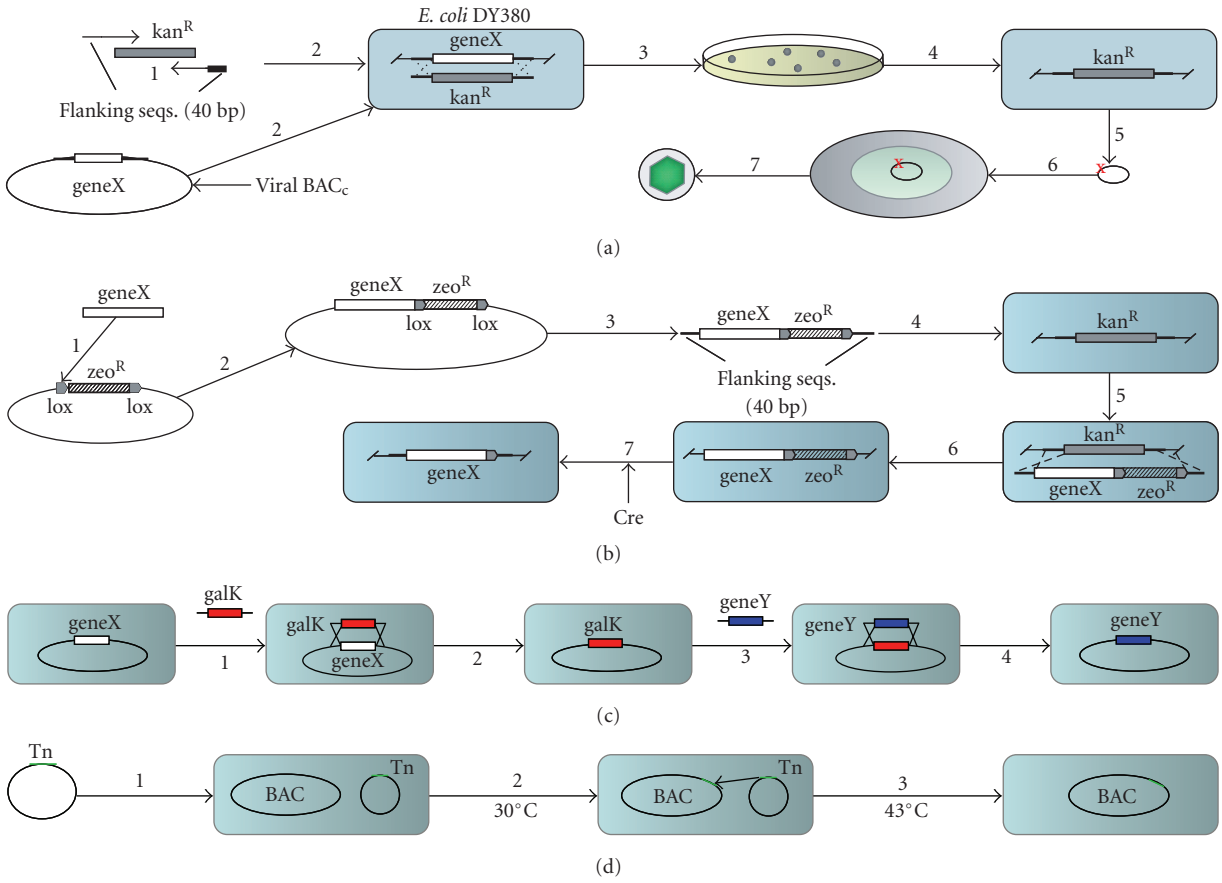

FIGURE 3: BAC mutagenesis techniques. (a) generation of a deletion mutant via homologous recombination. (1) Amplification of the kan^R expression cassette by PCR using a primer pair adding 40-bp (or longer) homologies flanking ORFX. (2) Viral BAC DNA is introduced into DY380 by electroporation. (3) Homologous recombination between upstream and downstream homologies of Gene X replaces Gene X with a selectable marker (the Kan^R cassette), creating the Gene X-deletion viral BAC. (4) The recombinants are selected based upon their ability to grown on LB agar plates containing kanamycin. (5) The viral BAC DNA is isolated, and the deletion of Gene X is confirmed by PCR analysis. The integrity of the viral genome (after homologous recombination) is examined by restriction enzyme digestion. (6) Purified BAC DNA is transfected into a human cell line. (7) Viral proteins are expressed, and a functional virus is created. B. Generation of Rescue Virus. 1. Gene X is amplified by PCR from the wild-type BAC DNA. (2) Gene X is cloned into a bacterial plasmid. (3) Gene X and a selectable maker (zeocin) are amplified via PCR using a primer pair that adds at least 40 bp of nucleotide sequence that is homologous to viral genomic sequence flanking Gene X. (4) The PCR product was transformed via electroporation into DY380, now carrying the Gene X deletion mutant. (5) and (6) Gene X (with a zeocin marker) is inserted back into the BAC by homologous recombination (7) The Zeo^R vector sequence is removed (by cotransfecting a Cre recombinase-expressing plasmid with the prepared viral BAC DNA). Rescue virus DNA is ready to be purified and transfected into human cells. (c) GalK-based Mutagenesis (1). Insert galK sequence flanked by a sequence homologous to the viral BAC sequence flanking Gene X. (2) Gene X is replaced by the galK gene via homologous recombination. (3) Replace the galK gene with PCR product containing desired mutation in Gene X (referred to as Gene Y). (4) Transfect viral BAC into mammalian cells to produce infectious mutant virus. (d) Transposon Mediated Mutagenesis. (1) and (2) A temperature-sensitive plasmid donor containing transposon (Tn) is inserted into *E. coli* cells already containing viral BAC that was inserted via electroporation). Once the donor plasmid is inside the cell, the transposon will be inserted into the BAC genome. (3) An increase in temperature will remove the donor plasmid. The transposon mutant is now ready to be purified and transfected into human cells. PCR primers pre-engineered into the transposon insertion site can be used to sequence the insertion region of any transposon mutants with interesting phenotypes.

Once colonies containing the galK gene are selected, the *galK* gene can be replaced with a PCR product that contains a modified sequence for the gene of interest. The bacteria harboring BAC DNA with the *galK* gene will not grow in minimal medium containing 2-deoxy-galactose (DOG) because the digestion of DOG produces toxic products. Therefore, colonies containing the modified gene sequence can be quickly selected due to negative selection of colonies with galK, which makes this time-saving system also highly efficient.

The method for creating rescue viruses using the *galK* system is identical to the procedure outlined above. In other words, the modified gene sequence is again replaced by the *galK* gene, which can then be replaced a PCR sequence for the original, unmodified gene sequence. Transfection of viral BAC into mammalian cells can produce an infectious rescue

mutant virus. This method for producing rescue viruses is much simpler than the method described for site-directed mutagenesis, which utilizes a positive selectable marker that requires the addition of a new foreign DNA for excision of unwanted sequence. However, this method also has certain disadvantages, such as the tendency for BACs to accrue undesired mutants. Although we have described negative selection using galK, there are also other negative selectable markers such as RpsL/Neo [73], SacB [74], and TolC [75].

3.4. Random Transposon Mutagenesis of Viral BACs. Transposon mutagenesis is another popular method for manipulating herpesvirus BACs [10, 13]. Transposons are mobile genetic elements that insert themselves into genomic DNA at essentially random locations, although different classes of transposons can have preferences for certain insertion site sequences. For example, random mutagenesis of viral BACs can be accomplished using a donor plasmid (such as a Tn1721-derived insertion from a modified TnMax plasmid [42, 43, 76–78]) with a transposon that has a preference to be inserted into plasmid (rather than genomic) DNA [10]. This insertion preference is useful because transposon insertion is a relatively rare event (each mutant should typically only have one mutation per viral BAC), so preferential transposon insertion into viral BACs will save a considerable amount of time by preventing transposon insertion into the *E. coli* genome.

The process of transposon mutagenesis is outlined in Figure 3(d). Briefly, a transposon donor plasmid is first transformed into *E. coli* cells containing viral BACs in order to allow insertion of the transposon into the viral BAC. If the donor transposon sequence is present on a temperature-sensitive plasmid, then temperature-sensitive transposon donors can be eliminated by a shift in incubation temperature so that the donor plasmid can no longer replicate. The transposon cannot further replicate without the donor plasmid because the transposon needs specific genes in order to replicate, which genes are provided by the donor plasmid. For example, Tn1721 requires transposase (e.g., *tnpA*) and resolvase (e.g., *tnpR*) genes in order to replicate itself [42]. Transposase cuts donor and genomic DNA, inserting the transposon into the viral BAC (along with the donor plasmid). Resolvase enables homologous recombination between inverted repeats (flanking the transposon sequence), thus allowing removal of the donor plasmid sequence and replication of the transposon sequence (now present both in the donor plasmid and the viral BAC).

If the transposon mutant shows an interesting phenotype during the infection of human cells, then the location of the transposon insertion must be determined. Transposons can be engineered so that flanking PCR primers that can be used to easily sequence the region of insertion can be inserted [42]. There are other slightly different methods for transposon mutagenesis [10]. The primary advantage to transposon mutagenesis is the speed with which a large number of mutations can be produced in a number of different BAC mutants. The disadvantages are: (1) it can be difficult to locate the insertion site without specially engineered transposons that contain PCR primer binding sites for insertion site sequencing, (2) it takes a long time to mutate every ORF because some ORFs will be "hit" multiple times while others will not hit at all, and (3) the results garnered by this method may be unclear (e.g., insertion in the middle of an ORF may produce a partial yet functional protein).

4. Studies of Specific Herpesvirus BACs

One of the first human herpesvirus BACs was produced for the herpes simplex virus (HSV-1) [17–19]. There have been a large number of studies focusing on mutations in one or two HSV-1 genes [79–84]. For example, one highly cited study demonstrated that HSV-1 gene ICP0 acts as an E3 ubiquitin ligase that can target cellular proteins for degradation [85]. Viral BAC technology made the genetic manipulations in this experiment feasible. There are also viral BACs for the other major strain of the herpes simplex virus (HSV-2) [20, 86], and both of these strains have been used for HSV-2 mutagenesis studies. Pseudorabies virus (PrV) is an alpha-herpesvirus that serves as a general model for herpesvirus pathogenesis, especially HSV-1. The PrV BAC was among one of the first nonhuman herpesvirus BACs to be constructed [46], and several PrV BAC mutants have been created and analyzed [71, 87–89].

Human cytomegalovirus (HCMV) is the largest human herpesvirus; it encodes around 165 ORFs (although the exact gene count varies over time) and grows slowly in culture [90]. Thus, BAC technology has been especially important for genetic studies of HCMV. The first BAC for HCMV was produced around the same time as the first HSV-1 BAC [25]. In fact, eight strains of HCMV have been cloned into viral BACs [26–29]. Each of these HCMV BAC strains is useful for different studies due to their similarity to "wild type" viruses that cause clinical infections or ability to grow in specific cell lines. For example, AD169, Towne, and TB40 are considered the "clinical" BAC strains whereas Toledo, FIX, PH, and TR are considered to be the "laboratory" BAC strains [28]. The eight HCMV BAC strains also vary in the number of generations that the parental virus was grown in cell culture prior to creation of a viral BAC, which is important because that means that he BACs will vary in similarity to the original clinical strain of virus. The self-excising AD169 BAC is also unique because it is the only HCMV BAC contains the entire genome of a clinical HCMV strain without containing the BAC vector [50]. Lack of the BAC vector is important because all other strains have a disruption of multiple US genes due to insertion of the BAC vector.

There have been a number of individual studies of HCMV BAC mutants [27, 91–93], but HCMV is unique in that multiple global analyses have also been performed. Global mutagenesis studies have utilized both random transposon mutagenesis [77, 94] and knockouts created via homologous recombination [52]. However, all these global deletion studies do provide global identification of essential and nonessential genes. For example, Hobom et al. sequenced only the HCMV glycoproteins following

global transposon mutagenesis [77]. Nevertheless, this study indicated that gB, gH, gL, and gM are essential for viral replication, but gp48 and gO are not (although the deletion of gO results in a severe growth defect). These findings are in complete agreement with later global studies [52, 94]. Global studies of herpesvirus gene function that were conduced in order to identify essential and nonessential genes are discussed in the next section.

There are also multiple animal models used to study HCMV pathogenesis. For example, there are BAC mutants for murine cytomegalovirus (MCMV [12, 51, 95–97]), rhesus cytomegalovirus (RhCMV [47, 98, 99]), and guinea pig cytomegalovirus (GPCMV [38, 100, 101]). There was also a large scale transposon mutagenesis study for MCMV [42, 43], which was the first global mutagenesis study conducted on any herpesvirus [42].

The first BAC for Epstein-Barr Virus (EBV) was also produced around the same time as the first HSV-1 BAC [24], and several different EBV BAC mutants have been produced for specific functional analyses over the past decade [102–107]. For example, Anderton et al. studied the pathogenesis of Burkett's lymphoma using three EBV deletion mutants (EBNA3A, EBNA3B, and EBNA3C) in order to determine that EBNA3A and EBNA3C cooperate to repress Bim, a tumor-suppressor gene [103]. EBV is one of two human gamma-herpesviruses (KSHV is the other human gamma-herpesvirus). Gamma-herpesviruses are unique because they are oncogenic. Therefore, there are a number of popular animal models for gamma-herpesviruses that are used to study how gamma-herpesviruses influence the development of cancer. The two most common animal models of gamma-herpesviruses are herpesvirus saimiri (HVS) and murine gamma-herpesvirus 68 (MHV-68); BAC mutants have been produced for both of these viruses [39, 44, 108, 109]. In fact, there is a global mutagenesis study for MHV-68 [45].

Kaposi's Sarcoma-Associated Herpesvirus (KSHV) is an oncogenic gamma-herpesvirus, and several KSHV BAC mutants have been constructed and analyzed [32, 33, 110–115]. For example, Lukac et al. studied how transcriptional activator ORF50 reactivates viral replication following latency by utilizing BAC mutants with deletions in downstream genes as well as BAC mutants with tandem histidine tags added to ORF50 [116]. In addition to the general gamma-herpesvirus animal models described for EBV (HVS and MHV-68), a handful of rhesus rhadinovirus (RRV) BAC mutants have been used to study KSHV pathogenesis [48, 117]. In fact, researchers have created a RRV BAC mutant with an autoexcisable BAC vector by inserting the vector into terminal repeat region of the viral genome and taking advantage of endogenous TR-mediated homologous recombination [117], and this technology may be able to applied towards creating traceless human herpesvirus BACs.

Varicella zoster virus (VZV) is the causative agent of chickenpox and shingles. Several studies have utilized VZV BACs to study the role of specific VZV genes [21–23, 59, 118], and there has also been a recent global mutagenesis study to determine which VZV genes are essential for viral replication [53]. (For more information about global analysis of gene function in VZV, see the next section.) VZV is the smallest human herpesvirus, and it replicates more quickly than some of the larger herpesviruses [4]. These features helped facilitate the creation of a luciferase reporter gene in a VZV BAC, which was the first BAC with a complete herpesvirus genome to utilize a luciferase reporter to help study viral replication *in vivo* [22, 59]. The use of luciferase markers to study viral replication is also discussed in the "Methods for BAC Construction" section of this paper, under the topic of "BAC Visualization Markers."

Human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) are beta-herpesviruses (similar to HCMV). An HHV-6 viral BAC has been constructed and HHV-6 BAC mutants produced to study HHV-6 pathogenesis [31, 119]. However, it should be noted that the HHV-6 BAC has not been sequenced, and the HHV-6 BAC is not currently independently infectious due to the size of the BAC vector (although this functionality should be present in a future viral BAC). No viral BAC has been constructed for HHV-7.

BAC mutants have also been used to study the herpesviruses that cause pathogenesis in animals. Examples include bovine herpesvirus type 1 (BHV-1 [34, 120, 121]), equine herpesvirus type 1 (EHV-1 [36, 122–124]), feline herpesvirus (FHV-1 [37, 125, 126]), koi herpesvirus (KHV [40, 127]), Marek's disease virus (MDV [41, 128, 129]), and turkey herpesvirus (HVT [49]). In fact, a global mutagenesis study has been conducted on BHV-1 [35], and a transposon mutation study has been conducted on EHV-1 [130].

5. Global Studies of Gene Function Using Human Herpesvirus BACs

Global studies of herpesvirus gene function are useful because they can provide lists of essential and nonessential genes for future studies [52, 53, 77, 94]. For example, the discovery of new essential genes may reveal an appealing novel drug target. Global studies on multiple cell and tissue types can also reveal tissue-tropic virulence factors [52, 53]. Genes that show severe growth defects in certain tissues may be appealing targets for mutagenesis in order to create a live attenuated vaccine vector. Findings from global human herpesvirus studies are outlined in Table 3.

HCMV is unique in that it is the only human herpesvirus to have multiple global deletion studies for genome-wide gene annotation. There are some discrepancies between the two HCMV global annotation studies, but at least some of these differences may be the results of subtle differences between the HCMV strains (one study utilized the AD169 BAC [94]; whereas the other used the Towne BAC [52]). Genome annotations fluctuate over time, so global mutagenesis studies carried out at different time may include slightly different sets of genes (e.g., Dunn et al. [52] provide annotations for 152 genes whereas Yu et al. [94] provide annotations for 156 genes). Methods for defining growth defect cutoffs were also slightly different, so this may also help explain differences in the number of nonessential genes and genes whose deletion causes a growth defect (referred to as "augmenting genes" in Table 3). Overall, the number of essential genes is similar for these two studies, and both

studies have similar broad conclusions, such as the tendency for essential and nonessential genes to cluster close to one another on the genome. One notable difference is that Dunn et al. [52] report four genes whose deletion causes enhanced growth (referred to as "repressive genes" in Table 3) but Yu et al. [94] do not report any such genes. Instead, all the genes whose deletion mutants caused enhanced growth are identified as nonessential in the latter study. Therefore, deletion of these genes could have caused an increase in growth rate in both studies. Nevertheless, the authors of the latter study might have simply decided that these genes were exhibiting wild-type growth, especially since it can be hard to assess statistical significance for these studies. In general, it is important to remember that these global studies usually examine phenotypes only in one or two cell types (or tissue types, in the case of VZV), and one deletion could interfere with the expression of neighboring genes. Of course, in-depth analysis of viral mutants produced in these global studies will require the mutants that are produced (in these studies) to be examined in other cell and tissue types.

Aside from HCMV, VZV is the only other herpesvirus to have annotations of essential and nonessential genes provided from a study of genome-wide mutagenesis [53]. The global study of gene function in VZV is also unique in that growth phenotypes were examined both in cultured human MeWo cells and in skin organ culture whereas HCMV global studies only examined phenotypes in cultured fibroblasts [52, 94]. This experimental design allowed for the discovery of skin-tropic virulence factors, three of which (ORF10, ORF14, and ORF47) were previously established in the literature, and one of which (ORF7) was a novel discovery presented in this study. In general, conducting global mutagenesis studies in other herpesviruses is likely to be very useful. For example, HCMV and VZV have a similar number of essential genes (between 41 and 45), but HCMV has twice as many genes as VZV. Conserved genes are slightly more likely to be essential, but all of the essential genes do not necessarily have high sequence similarity between human herpesviruses [53]. Therefore, it would be interesting to see whether all the human herpesviruses have a similar number of conserved essential genes and determine if these genes always share a common biological function (such as enrichment with genes related to DNA replication).

6. Other Herpesvirus BAC Applications

In addition to the local and global mutagenesis studies described in the previous section, BAC mutants can serve as a vector for genomic elements that can facilitate the study of herpesvirus pathogenesis [131–133]. Gene modifications (frame-shift mutations, premature stop-codon mutants, point mutations, etc.) can be created using homologous recombination, which requires two basic steps. First, PCR primers for sequences flanking the gene of interest are designed as well as primers that contain the mutated region of the gene of interest. Regions of the gene before and after the mutation are amplified via PCR. For the second step, these two fragments are combined and amplified via PCR reaction, using the primers flanking the sequence for the original gene. This mutated gene sequence can then be inserted into the BAC using homologous recombination, similar to the method by which BAC deletion mutants are created. Larger modifications can be created using similar methods. For example, it may be useful to insert an epitope tag (e.g. V5) into a gene sequence for a gene without an existing antibody [70, 94, 134]. Chimeric genes recognized by epitope antibodies can facilitate biochemical analysis for genes of unknown function. Likewise, a similar method can be used to create green fluorescent protein (GFP) fusion mutants. This technology was first developed for use in a PrV BAC to study the US9 gene [71]. In general, GFP-fusion mutants can be useful for studying the expression and localization patterns of viral genes [135–138]. Protein complementation assays (PCAs) can also be used to test protein-protein interactions (and conservation of gene function between herpesviruses) between two gene fragments inserted into a BAC [139]. New technologies have also allowed for the creation of conditional deletion mutants that contain destabilizing domains that can be stabilized by addition of addition of the synthetic ligand (called shield-1), thus allowing for knockout studies of essential genes [72]. This technology can also be useful for vaccine development because vaccine strains can be grown *in vitro* with addition of shield-1 but the strain will be unable to replicate when injected into patients due to the lack of shield-1.

Another exciting area of herpesvirus BAC research is the potential development of novel vaccines. BAC-based vaccine candidates have been developed for HCMV [100, 140], HSV-1 [141, 142], and VZV (H. Zhu, unpublished data). An MCMV vaccine was also developed as a proof of concept [95, 143], and a live-attenuated guinea pig cytomegalovirus vaccine has been shown to be immunogenic [101]. Inoculation of RhCMV in rhesus macaques has shown that secondary RhCMV infections require inhibition of MHC-1 antigen presentation, and this finding may be helpful in designing an effective HCMV vaccine [144]. An EHV-1 BAC has also been used as a vector to induce an immune response to produce West Nile Virus antibodies [145], express bovine diarrhea virus structural proteins [146], and immunize mice against Venezuelan Equine Encephalitis Virus [147].

HSV-1 BACs are also employed in gene therapy treatments [142]. HSV-1-based gene therapy vectors contain the viral genes necessary for viral replication, but lack the virulence factors necessary to cause a clinical infection. For example, HSV-1-based gene therapy vectors have been developed in the hopes that they will be effective in treating cancer [60, 148] and osteoporosis [149]. EBV BACs have also been used as gene therapy vectors in some situations [61, 150].

In conclusion, the amount of biomedical research utilizing herpesvirus BACs has grown rapidly during the past decade, resulting in invaluable knowledge about viral pathogenesis, vaccine development, and gene therapy. Since the construction of the first herpesvirus BAC 12 years ago, BACs have been generated for all major human and animal herpesviruses, and this technology has greatly facilitated genetic and functional studies of herpesviruses

because recombinant herpesviruses were previously difficult to produce due to their large size. Soon, we may have BACs for all the human herpesviruses as well as novel global mutational studies for several herpesvirus BACs. Global and local studies of herpesvirus pathogenesis should help identify new antiviral targets and produce more effective and safe vaccines. For example, additional mutagenesis studies may be useful in revealing tissue tropism factors and/or novel viral-host interactions. In short, future herpesvirus BAC studies should help provide exciting new discoveries about viral pathogenesis as well as therapeutics for both viral and nonviral diseases.

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