



Allele replacement: an application that permits rapid manipulation of herpes simplex virus type 1 genomes

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Herpes simplex virus (HSV) is a new platform for gene therapy. We cloned the human herpesvirus HSV-1 strain F genome into a bacterial artificial chromosome (BAC) and adapted chromosomal gene replacement technology to manipulate the viral genome. This technology exploits the power of bacterial genetics and permits generation of recombinant viruses in as few as 7 days. We utilized this technology to delete the viral packaging/cleavage (pac) sites from HSV-BAC. HSV-BAC DNA is stable in bacteria

and the pac-deleted HSV-BAC (p45–25) is able to package amplicon plasmid DNA as efficiently as a comparable pac-deleted HSV cosmid set when transfected into mammalian cells. Moreover, the utility of bacterial gene replacement is not limited to HSV, since most herpesviruses can be cloned as BACs. Thus, this technology will greatly facilitate genetic manipulation of all herpesviruses for their use as research tools or as vectors in gene therapy.

Keywords: HSV-1; gene replacement; amplicons; bacterial artificial chromosome

Introduction

Herpes simplex virus (HSV) is the prototypic human herpesvirus. The study of viral genetics has resulted in a considerable body of information on the biology and molecular biology of HSV. Generation of HSV mutants has often relied upon drug selection or cotransfection of cells with intact viral and plasmid DNA, usually modified by insertion of a marker gene. Viral mutants containing either point mutations, insertions or deletions are identified by selecting for resistance to drug selection, screening for expression of the plasmid marker, screening for viability in complementing cell lines, or by direct structural characterization.^{1–4} Another way of generating HSV mutants is to use cosmids comprising the HSV genome. Cosmid sets that contain the genomes of the human herpesviruses, VZV, HSV, CMV and EBV have been constructed.^{5–8} Viral plaques are produced by transfecting the cosmid sets into mammalian cells, which results in recombination between the overlapping fragments thereby reconstituting the viral genome.

Specific mutations can be readily introduced into the genome by manipulating cosmid DNA.^{5–8} For example, Cunningham and Davison⁶ constructed mutant viruses that were deficient in U_L2 or U_L44 by manipulating an HSV cosmid set. Fraefel *et al*⁹ inactivated the cleavage/packaging function by deleting the 'a' (*pac*) sequences from the same HSV cosmid set. Transfection of this reagent and an amplicon plasmid, containing HSV ori_s and a *pac* site, into mammalian cells resulted in packaging of only amplicon plasmid DNA into viral particles.

These helper-free amplicons have great utility in gene therapy.^{9–11} However, maintenance of HSV cosmid sequences in *E. coli* can be problematic due to lack of genetic stability (Refs 6, 12; this report).

To overcome these problems, Messerle *et al*¹³ cloned MCMV as a BAC and Stavropoulos and Strathdee¹² cloned an HSV strain 17 BAC that was reverse engineered from *pac*-deleted cosmids. However, HSV-BAC generated in this way does not replicate. To create a replication-competent HSV-BAC, we cloned the human herpesvirus HSV-1 strain F genome into a single bacterial artificial chromosome, and tested a method that permits rapid generation of viral mutants using bacterial genetics. We describe the construction of a *pac*-deficient HSV mutant and demonstrate the ability to generate amplicons. This technology will greatly facilitate manipulation of HSV and potentially other herpesviruses for their use as research tools and as vectors in gene therapy.

Results

Generation of recombinant viruses and BAC plasmids
We used bacterial artificial chromosome (BAC) technology to clone the HSV genome as a single molecule. As a first step, we created an HSV recombinant that contained BAC and marker sequences. We decided to introduce the BAC sequences into the *tk* locus. pBAC-TK, was constructed with viral *tk* sequences flanking the signals necessary for chromosomal maintenance in bacteria¹⁴ and the chloramphenicol (*cm*) resistance gene. This plasmid was cotransfected with infectious HSV-1 DNA into Vero cells and the resultant virus stocks screened with 100 µm ACV to isolate drug-resistant viruses. A recombinant virus containing BAC and *cm* sequences within the *tk* locus, HSV-BAC, was isolated and plaque purified.

To isolate circular DNA for subsequent transformation into *E. coli*, Vero cells were infected with HSV-BAC for 2 h and harvested. Isolated circular DNA was electroporated into *E. coli* and recombinant colonies resistant to *cm* were subjected to further analysis. Bacterial colonies were subjected to PCR analysis using primer sets specific for HSV genes US6, UL10, UL30 and UL40. Eleven of 11 clones tested were positive for all four primer sets. These results suggested that the genome of HSV had been cloned successfully.

Rescue of infectious virus progeny from HSV-BAC clones

One clone, p25, was selected for further analysis. Transfection of p25 into Vero cells resulted in plaque formation after 36 h and complete destruction of the monolayer by 3 days. This virus, r25, was amplified and subjected to further analysis.

To test whether passage of HSV sequences through bacteria was detrimental, the growth rates of the parental input virus and r25 were compared. One-step growth curve experiments revealed that there were no significant differences in the growth rates of these viruses over time in Vero cells (Figure 1a). Furthermore, the banding patterns of *EcoRV*-digested DNA isolated from HSV-BAC, r25 and p25 were identical (Figure 1b and data not shown). Thus, it appeared that the HSV genome contained within the BAC was stable when passaged in bacteria.

BAC plasmids are stable in bacteria

Maintenance of HSV sequences in *E. coli* can be problematic; the genome contains palindromic sequences that are often unstable in bacteria, eg Ori_L.¹⁵⁻¹⁷ To assess stability of HSV-BAC clone p25, we isolated p25 DNA from bacterial cultures serially passaged every 24 h for 7 days

(Figure 2a). The restriction pattern of p25 remained unchanged throughout this period. By contrast, the restriction patterns of cosmid clones, containing HSV sequences, 6, 14, 28, 58 and 48⁶ were altered significantly. A representative sample is shown in Figure 2a. Furthermore, we often detected alterations in DNA profiles after only 12 h of passage (data not shown). However, Cunningham and Davison⁶ had shown that heterogeneity existed in cos 28 isolates. This was due to the instability of Ori_L sequences. To ascertain whether Ori_L sequences were stable in HSV-BAC, we digested clone p25 or cos 28 with *KpnI* (Figure 2b). We could not detect any heterogeneity in the HSV-BAC fragment containing Ori_L (*Kpn* v), indicating that this sequence was stable. Heterogeneity in the bacterial population was assessed by characterizing DNA from five p25 clones and six cosmid 56 clones (Figure 2c)⁶ by digestion with restriction enzymes. Results demonstrated that BAC clones in *E. coli* were homogeneous whereas cosmid clones were heterogeneous. However, we could not exclude the possibility that HSV-BAC plasmids contain undetected mutations that may be transferred to viral progeny. Nevertheless, it is likely that maintenance of HSV sequences in BACs will be preferable over cosmids for production of reproducible infectious viral DNA for therapeutic or experimental purposes.

Manipulation of BACs containing HSV sequences

We adapted technology developed for chromosomal gene replacement in bacteria;¹⁸ to permit rapid manipulation of BAC sequences. A vector was constructed, pKO5 (Materials and methods), that contains a replacement allele, a temperature-sensitive origin of DNA replication, a marker for positive selection in bacteria, zeocin, and a marker for negative selection, *SacB* (*SacB* encodes levansucrase, which is lethal when expressed in *E. coli* growing

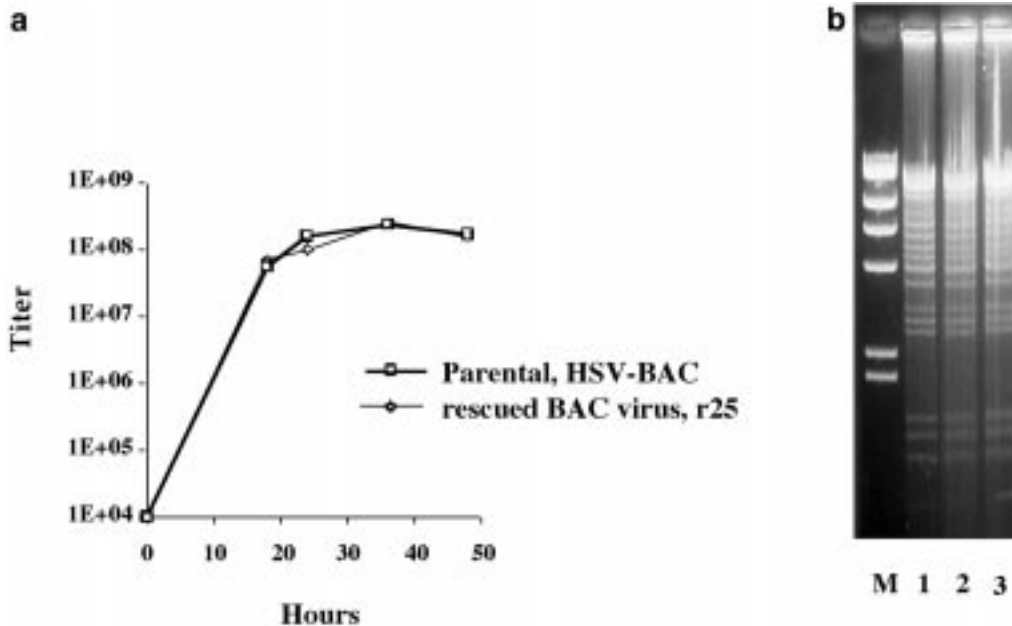


Figure 1 (a) Rescue of infectious progeny from HSV-BAC clones. HSV-BAC DNA was transfected into Vero cells and virus, r25, isolated. One-step growth curves between r25 and the parental recombinant virus, HSV-BAC showed no significant difference in the ability of these viruses to grow over time. (b) *EcoRV* restriction enzyme digests of: (1) HSV-BAC DNA; (2) r25; (3) p25. (M), lambda DNA *HindIII* markers.

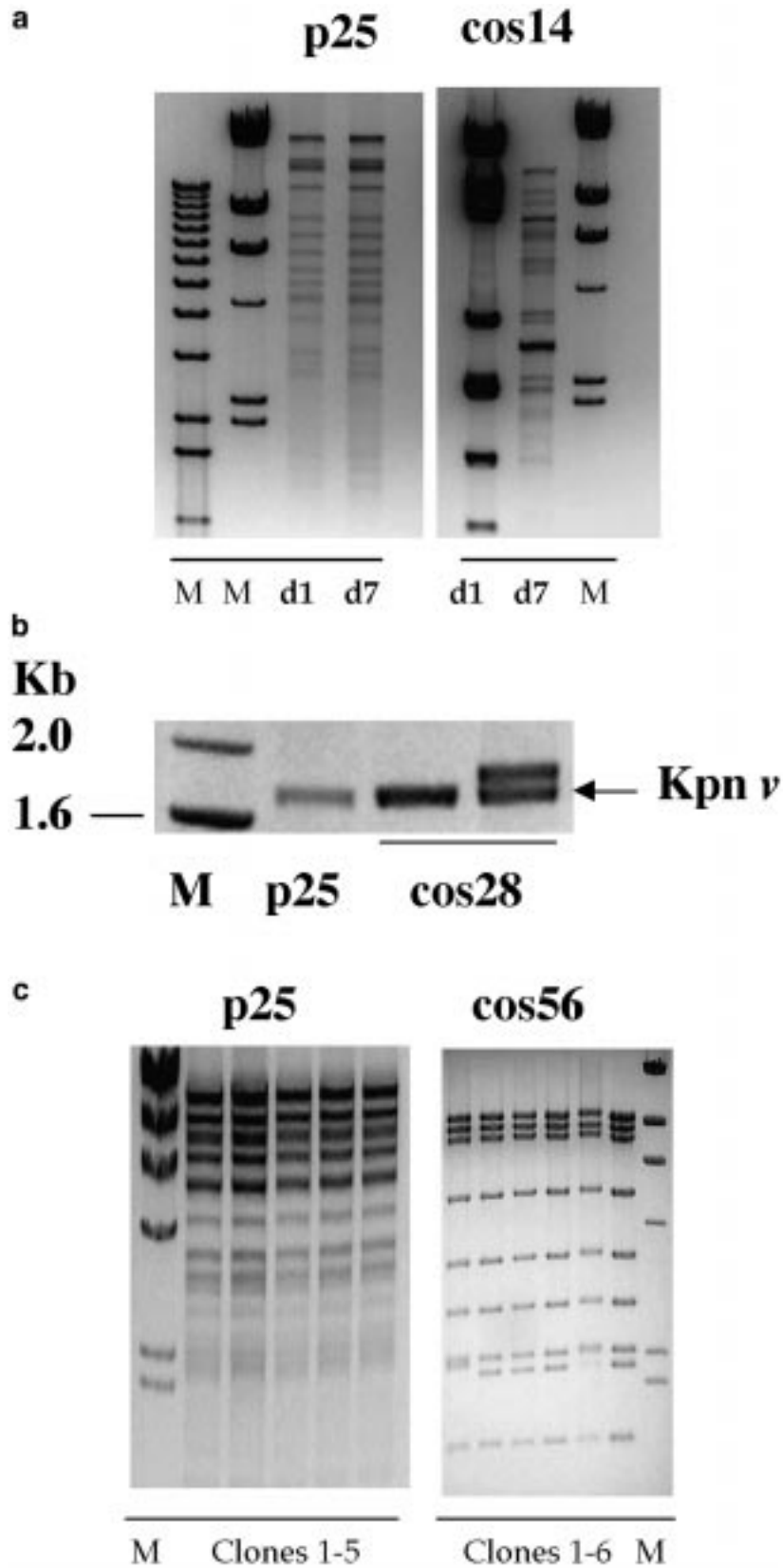


Figure 2 DNA stability and homogeneity studies. (a) p25 or cosmid 14⁶ containing *E. coli* were serially passaged for 7 days. DNA was isolated at days 1, 3 (not shown) and 7, and digested with EcoRV. M, Kb ladder or lambda DNA HindIII. HSV-BAC DNA does not alter with time. (b) KpnI digest of p25 or two isolates of cos 28 DNA. The Kpn v fragment, containing Ori_i, is marked with an arrow. (c) p25 and cosmid 56⁶ containing *E. coli* were streaked out on to LB agar plates containing the appropriate antibiotics. DNA was isolated from single isolates and digested with either XhoI or BamHI, respectively. M, lambda DNA HindIII. HSV-BAC isolates are homogeneous.

on media supplemented with 5% sucrose). Figure 3 illustrates and describes the mutagenesis procedure. Briefly, the *in vitro* altered sequences are transformed into *E. coli* and co-integrates (that arise as a result of homologous recombination), are selected by plating cells on cm/zeocin plates at the nonpermissive temperature (43°C). Although the plasmid can integrate into either the BAC or the *E. coli* chromosome, it is expected that HSV sequences used to target the plasmid will favor HSV-BAC integration. To select for excision and loss of the plasmid, co-integrate colonies are diluted and plated on LB plates containing 5% sucrose at the permissive temperature (Figure 3). At the permissive temperature, the plasmid origin is unstable in the BAC, and the plasmid is excised by homologous recombination at either cross-over point 1 or 2 (Figure 3). This can result in exchange of the target allele with the mutant allele. Cells that lose the plasmid containing *SacB* sequences are able to grow under these conditions. Sucrose and cm-resistant colonies are screened for the desired gene replacement event by PCR.

To ascertain whether HSV-BAC sequences were altered by the heatshock procedure, *E. coli* harboring HSV-BAC were subjected to growth at the nonpermissive temperature and/or sucrose selection and DNA isolated. Extensive restriction enzyme digestions revealed a small deletion in DNA that had undergone heatshock (data not shown). To characterize the deletion, we subcloned and sequenced the appropriate DNA fragments isolated from bacteria that had been incubated at either the permissive or nonpermissive temperature. The deletion extended for

500 bp 5' of nucleotide 118 565 relative to the strain 17 sequence.¹⁹ However, the deletion did not result in loss of any known regulatory elements and further, rescued virus grew as well as wild-type virus (Figure 4). Repetition of the process did not result in any further DNA changes. It is likely that this HSV strain F sequence was toxic to *E. coli* under heatshock conditions.

Deletion of the pac sites from HSV-BAC clone, p25

To illustrate the power of bacterial genetics in manipulation of HSV genomic sequences, we chose to generate HSV-BAC constructs suitable for amplicon vector production by deleting both *cis* HSV *pac* sites from p25. HSV sequences 120 902–125 769 and 126 774–131 249 were cloned into the unique *Sa*II site of pKO5, creating pKO5-LARA (Materials and methods; Figure 5a). This construct lacks HSV sequences, 125 770–126 773, that contain the *pac* site. pKO5-LARA was transformed into *E. coli* containing p25 and plated on LB plates containing cm and zeocin at the nonpermissive temperature. To select for excision and loss of plasmid, six co-integrate colonies were diluted and plated on LB plates containing 5% sucrose at the permissive temperature then replica plated on to either cm or zeocin plates to confirm the presence of BAC and loss of plasmid sequences (Figure 5b). PCR, using primers that flank the putative deletion demonstrated that *pac* sequence deletions were present in approximately 30% of the BAC clones (Figure 5c and data not shown). The deletions were confirmed by Southern blotting (data not shown). One clone, p45, was picked for further analysis. Restriction enzyme digests indicated

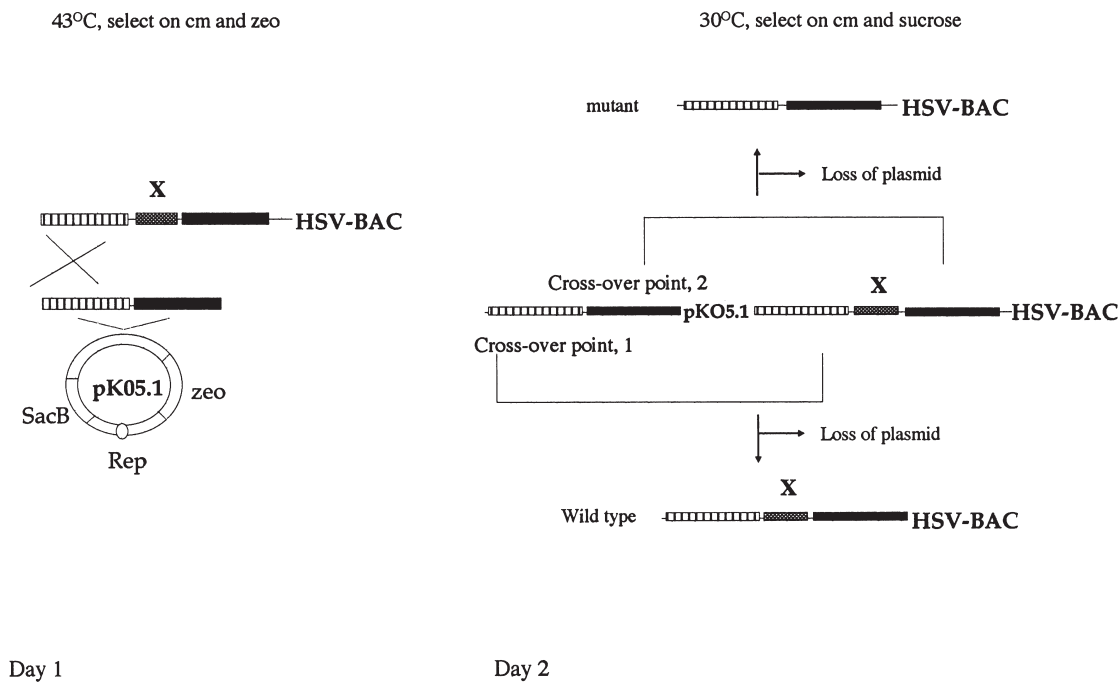


Figure 3 A diagram illustrating the gene replacement vector and procedure to delete gene *X* from HSV-BAC. (Day 1) The KO5 vector carrying the altered allele is transformed into *E. coli* harboring HSV-BAC and plated at the nonpermissive temperature on cm/zeo LB plates. In this example, the object is to delete gene *X* from HSV-BAC. *SacB*, *zeo* genes and the temperature-sensitive origin of replication, *Rep*, are indicated. Co-integrates that arise via homologous recombination are able to survive these conditions; an integration event allows replication of plasmid sequences by the BAC origin. (Day 2) When shifted to the permissive temperature, the plasmid origin is unstable in the BAC and is excised at either cross-over point 1 or 2 by homologous recombination resulting in exchange of allele at frequencies of up to 50%. The counter-selectable marker, *SacB*, is used to select for loss of plasmid sequences. Sucrose-resistant colonies are replica plated on to zeocin plates to confirm loss of plasmid sequences and the allele replacement event screened by PCR.

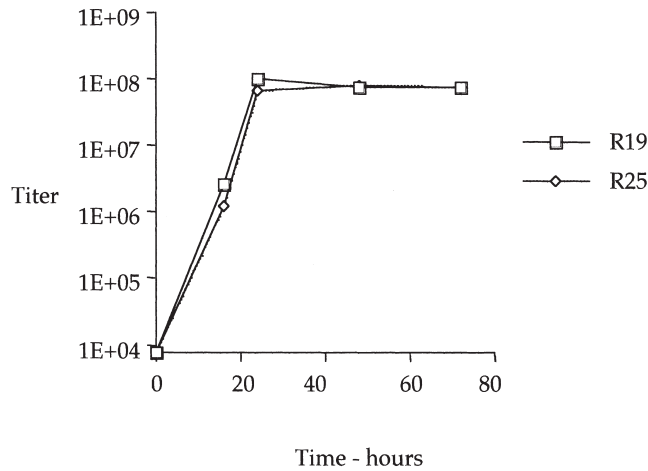


Figure 4 p25 DNA was transformed into RR1 cells, plated on to cm plates and incubated overnight at 43°C. DNA was extracted from colonies, transfected into Vero cells and infectious viral progeny, R19, isolated. Vero cells were infected at an MOI of 0.01 with either R25 (no heatshock) or R19 and plaques counted at 16, 24, 48 and 72 h.

that: (1) the internal *pac* sequence was absent from clone p45; and (2) the alteration in the *DraI* banding profile of p45 was exactly as predicted from sequence information (Figure 5d). The procedure was repeated to delete the remaining *pac* sequence from p45. Several clones, p45-12, p45-14, and p45-25, were identified and subjected to Southern blotting analysis. Results indicated that the mutant allele carried on the plasmid had replaced both BAC *pac* sequences (Figure 5e and data not shown). Furthermore, either *EcoRV* (Figure 5f), *EcoRI*, *Sall*, *SacI*, or *DraI* (not shown) restriction patterns of DNA extracted from p45, p45-12, p45-14 and p45-25, confirmed that BAC plasmids remained stable during the mutagenesis procedure.

HSV-BAC DNA can package amplicons

To test infectivity and plaque formation, we transfected either p25 or p45-25 into Vero cells. Wild-type HSV-BAC, p25, generated plaques 36–48 h after transfection whereas we could not detect plaques in p45-25-transfected cells despite passaging them for 28 days. *Trans* functions were tested by determining the ability of p45-25 (HSV-BAC Δ_2) DNA to package amplicon plasmid DNA. Amplicon plasmids containing *lacZ* under the HSV IE4/5 promoter were cotransfected with *pac*-deleted HSV sequences of either p45-25, or cosmid DNA.^{6,9} Cells were harvested after 4 days and infectious amplicon particles quantified by infecting fresh monolayers and counting blue cells or viral plaques (Table 1). The result of this experiment indicated that both HSV-BAC DNA and cosmid DNA packaged amplicons. However, we could not accurately quantify the p45-25 packaging efficiency, as in some instances we could detect helper virus that had arisen via recombination between the homologous sequences present in both p45-25 and the amplicon plasmid, that is, Ori_S. These data suggest that despite the advantage of DNA stability, HSV-BAC will need to be further modified before it becomes a useful vector for production of helper-free amplicons for gene therapy.

Discussion

Construction of viral herpesvirus mutants by conventional means can often be a lengthy process. Usually, the mutant allele is cotransfected into mammalian cells with infectious viral DNA, and recombinant viruses screened for by standard molecular techniques. Once identified, the recombinant virus needs to be plaque purified to remove contaminating nonrecombinant virus.

To facilitate the process of human herpesvirus genome manipulation, the genomes of VZV, HSV, CMV and EBV were cloned as cosmids.^{5–8} Several mutant viruses have been rapidly constructed by manipulating these cosmids.^{5–8} However, we and others have found that maintenance of HSV sequences in cosmids is not stable (Refs 6, 12; Figure 2a). Furthermore, as the starting material is heterogeneous (Figure 2c), the generated mutant viruses may contain unknown mutations at other sites. Thus, the properties of HSV preparations that are produced from cosmid DNA may vary from batch to batch. Obviously, this has important ramifications if HSV cosmid sets are used to generate helper-free amplicons for use in gene therapy.^{9–11}

To overcome the deficits of cosmid mutagenesis and to expedite and simplify the procedure of mutant virus construction, we adapted technology that was developed for gene replacement in *E. coli*.¹⁸ The procedure requires two reagents, an HSV-BAC and a gene replacement vector. The construction of the first reagent follows standard virological techniques and exploits the fact that the HSV genome circularizes in the nucleus of infected cells.^{20,21} Other herpesvirus BACs have been constructed. Messerle *et al*¹³ and Stavropoulos and Strathdee¹² have respectively cloned a murine cytomegalovirus genome as a BAC from infectious virus and an HSV strain 17 BAC that was reverse engineered from cosmids. The second reagent, the gene replacement vector, contains a mutant allele, either an insertion, deletion or point mutation. The vector is transformed into HSV-BAC containing bacteria and subjected to selection as outlined (Figure 3). Depending upon the length of the flanking sequence that is used for homologous recombination, these frequencies can occur approximately 50% of the time. Thus, as many as half of the sucrose-resistant colonies may contain mutant BAC sequences. PCR can be used to screen for the desired mutation at this stage as there are no plasmid sequences present in bacteria that could give a false PCR result (see Figure 5b). Once detected, BAC DNA can be harvested from bacteria and transfected into mammalian cells and virus recovered 2 days later. Taken together, this procedure allows generation and isolation of mutant viruses within 7 days. Moreover, all mutant vectors are clonally derived and isolated in the absence of wild-type virus; thus, there is no need to plaque purify recombinant viruses. However, we did observe that some HSV strain F sequences are toxic to *E. coli* at 43°C. These sequences, reiterated repeats upstream of the *lat* promoter, were deleted in bacteria. The number and nature of these repeats vary between and within HSV strains.^{22,23} However, analysis of the rescued virus indicated that there were no growth defects (Figure 4) and experiments are underway to address the behavior of the mutant virus *in vivo*. Nevertheless, once deleted, the HSV-BAC is stable and mutagenesis could be repeated without any other detectable changes. It is likely that use of this procedure

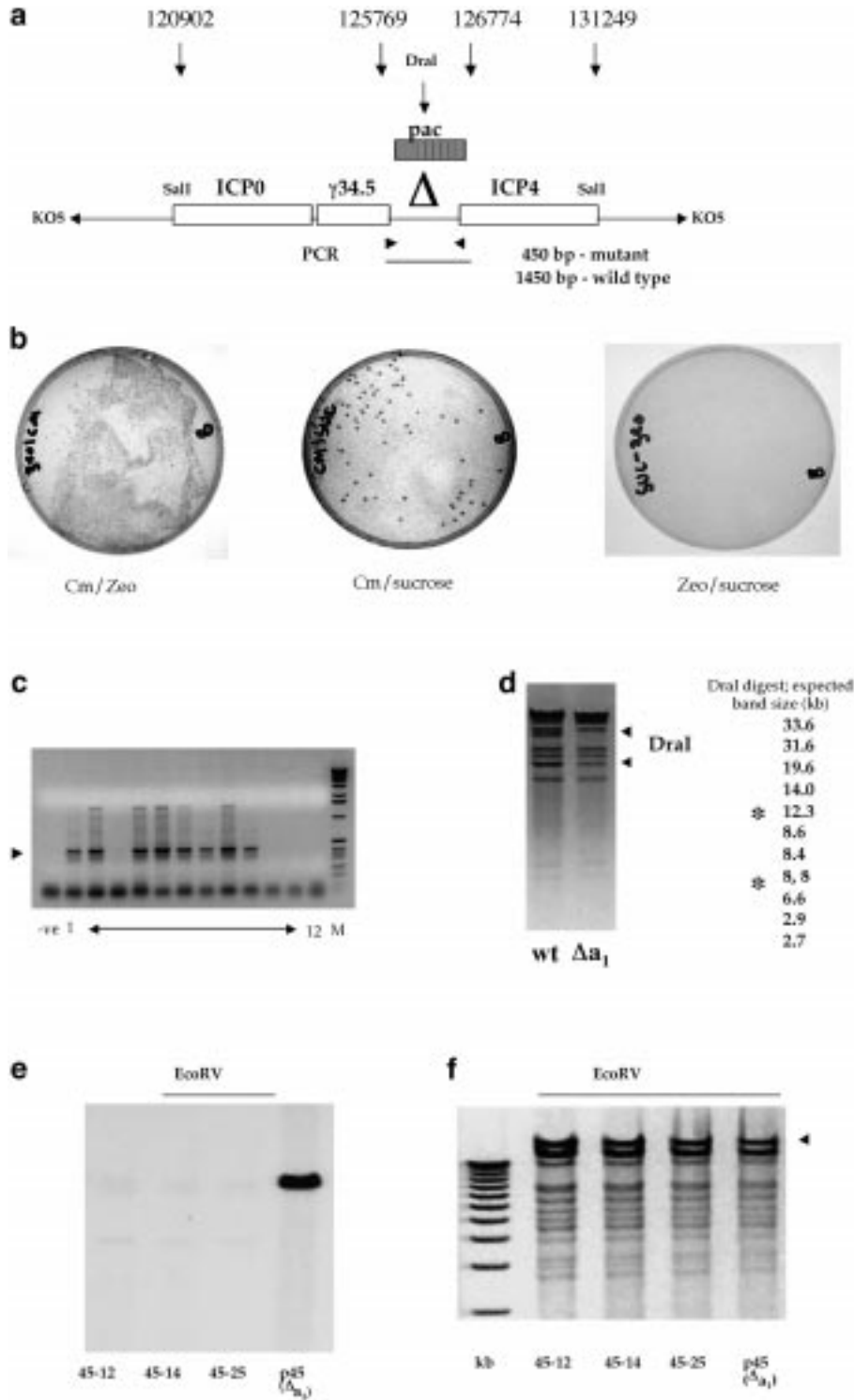


Figure 5 Deletion of the *pac* sites from HSV-BAC. (a) Schematic of pKO5-LARA. The coding sequences of ICP0, γ 34.5 and ICP4 are indicated as are the restriction sites, *Sall* and *DraI*. The nucleotide positions relative to strain 17 are indicated. This construct will delete the intervening *pac* sequence, indicated as a delta sign. Primers used for PCR are shown as black triangles. A black line represents a PCR product that would correspond to a deletion. (b) Serial dilution of a co-integrate colony that was plated on to LB agar plates containing cm/zeo or cm/sucrose or zeo/sucrose. (c) PCR results from a number of cm/sucrose-resistant, zeocin-sensitive colonies. In this example, eight of 12 colonies contained BACs that were deficient for one *pac* site. The PCR conditions that were employed were not optimal for production of the 1.5 kb wild-type product. -ve, water control. (d) A *DraI* restriction digest of p25 (wt) DNA and p45(Δa_1) DNA. Deletion of a *pac* site in p45 results in loss of a *DraI* site, ie loss of an 8 and 12.3 kb band, marked by arrows on the gel and as asterisks by the expected band sizes. (e) Southern blot of *EcoRV* digested DNA as shown in (f) probed with a radiolabeled *pac* fragment probe indicating that the progeny isolated from the second round of mutagenesis do not have *pac* sites. (f) *EcoRV* digest of DNA isolated from three colonies that were identified from the second round of mutagenesis. No unexpected alteration of banding pattern was detected, indicating that the mutagenesis procedure is reliable. The black arrow identifies the fragment that would contain the second *pac* deletion. This fragment is 40 kb, and the mutated fragment, 39 kb. This size shift cannot be detected on this gel.

Table 1 Packaging of an HSV amplicon vector

Helper construct	Vector ^a	Titer b.t.u./ml	p.f.u./ml
p25	pHSVlac	2e7	2e8
p45-25		NA	0 ^b
p45-25	pHSVlac	4e4	8e4
p45-25	pHSVlac	8e5	0
p45-25	pHSVlac	6e5	0
p45-25	pHSVlac	4e4	2e4
p45-25	pHSVlac	8e4	1e5
HSVΔa	pHSVlac	1e5 ^d	0
cosmids ^c			

HSV-BAC or cosmid DNA with or without the reporter amplicon plasmid, pHSVlac, was transfected into 2–2 cells, and harvested as described in Materials and methods. The experiment was repeated six times. Values are expressed as either blue transducing units (b.t.u.) per ml or as plaque forming units (p.f.u.) per ml.

^aGeller.²⁷

^bNone detected in a 28 day assay.

^cFraefel *et al.*⁹

^dAverage titer determined from six independent transfections. NA, not applicable.

in other bacterial strains could prevent this deletion from occurring, and this remains to be investigated.

To illustrate the ease with which we could manipulate the HSV genome, we deleted the *pac* sites of HSV-BAC. This reagent, p45–25, was able to package amplicon plasmid (Table 1). However, in several transfections, we could detect the presence of helper-virus. It is likely that the helper-virus arose as a result of recombination between the Ori_S sequences that are present in both the amplicon and HSV-BAC plasmids. However, the amounts of contaminating virus were low, ranging from undetectable to a ratio of 1 helper-virus: 1 amplicon. Generation of HSV-BAC-derived helper-free amplicons will require that there are no homologous sequences between amplicon and HSV-BAC plasmids. This could be accomplished by deleting the Ori_S sequences from p45–25; Igarashi and colleagues²⁴ determined that an Ori_S-deleted HSV-1 could replicate nearly as well as wild-type virus.

We chose to insert the BAC sequences into the *tk* locus, because we can readily select for insertion or deletion of BAC sequences. This strategy is useful as BAC sequences may be problematic if the mutated virus is to be used in a clinical setting. However, any viruses that are produced from our reagent will be TK negative and thus deficient for most animal studies. Nevertheless, it is relatively straightforward to introduce the BAC sequence to other loci in the HSV genome.

In conclusion, we have developed a new methodology that overcomes the deficits of cosmid technology. The BAC/KO5 system will greatly facilitate manipulation of HSV and other herpesviruses for investigating basic biological questions and for their use as vectors.

Materials and methods

Viruses and cells

Vero and Vero 2–2 cells²⁵ were propagated and virus assayed for acyclovir susceptibility as described by Hors-

burgh *et al.*²⁶ Virus titers were determined in triplicate on Vero cells. Recombinant viruses were plaque purified two times and the mutation(s) confirmed by Southern blot hybridization and PCR analysis.

Plasmids

Plasmid pTK-AB was created by subcloning the *Bgl*III/*Eco*RI fragment from pXholf, strain 17, (Chris Preston, University of Glasgow) into the *Bam*HI/*Eco*RI sites of pHSVlac.²⁷ The *tk* containing plasmid, BH13, strain 615.9,²⁸ was digested with *Pst*I and *Xho*I, to release the 3' end of the *tk* gene, and the fragment subcloned into the *Pst*I/*Xho*I site of pSC1180, creating plasmid TK-CD. Plasmids TK-AB and TK-CD were digested with *Hind*III/*Msc*I and *Hind*III/*Sal*I respectively and the fragments subcloned into the *Hpa*I/*Sal*I sites of pBelobac²⁹ to create plasmid pBAC-TK. pKO5 was generated by cloning the zeocin gene as a blunt-ended *Ssp*I-*Sty*I fragment into the *Nae*I site of pKO3¹⁸ and deleting the chloramphenicol (*cm*) gene by digesting with *Bst*BI and religating. Construction of pKO5-LARA was as follows. The *Bst*EII fragment containing HSV sequences 120 902–125 769 was blunt-ended and cloned into the *Eco*RV site of pBluescript (Stratagene, La Jolla, CA, USA) creating pLA. pK1–2³⁰ was digested with *Eco*RI and HSV sequences 126 774–131 249 were cloned into the *Eco*RI site of pLA creating pLARA. pLARA was digested with *Sal*I and the HSV containing sequences purified and cloned into *Sal*I digested pKO5.

PCR

PCR-amplified fragments were obtained using viral genomic DNA or plasmid DNA as template and the oligonucleotide primers listed below. *Taq* DNA polymerase (Promega, Madison, WI, USA) was used to amplify the region of interest with 30 cycles (95°C, 1 min, 55°C, 1 min, 72°C, 1 min) being performed in a Perkin Elmer Gene-amp 2400 (Norwalk, CT, USA). The primers that were used in this work are as follows.

Chloramphenicol 5'AGGCCGGATAGCTTGTGC and 5'CGGAACAGAGAGCGTCACA
 U_S6 5'CCGAATGCTCCTACAACAAG and 5'GTCTTCCGGGGCGAGTTCTG
 U_L10 5'GGTGTAGCCGTGCCCTCAG and 5'GCAGAT-ACGTCCCGCTCAGG
 U_L30 5'ATCAACTTCGACTGGCCCTTC and 5'CCGTA-CATGTCGATGTTCCACC
 U_L40 5'ACCGCTTCCTCTTCGCTTTC and 5'CCCGCA-GAAGGTTGTTGGTG

Virus construction

pBAC-TK was digested with *Hind*III and cotransfected into Vero cells with infectious HSV-1 strain F DNA as described by Chiou *et al.*³¹ DNA from plaques that were resistant to 100 μm ACV was screened by PCR using primers that correspond to chloramphenicol sequences (see above). From each of two independent transfections, two recombinant viruses were plaque purified two times and the mutation confirmed by Southern blot hybridization. One virus, HSV-BAC, was subjected to further analysis.

Isolation of HSV-BAC DNA and BAC plasmids

At a multiplicity of infection (MOI) of 3, 100 mm dishes of confluent Vero cells were infected with HSV-BAC.

Infections were allowed to proceed for 2 h, the supernatants removed and 1 ml of DNazole (Gibco, BRL, Burlington, ON, Canada) added. DNA, obtained from the cell lysate by following the manufacturer's protocol, was resuspended in 100 μ l of TE. One microliter of DNA was added to an equal volume of water and electroporated into 25 μ l of electro-competent *E. coli*, strain DH10B, using a cell-porator (Gibco) and conditions recommended by the manufacturer. The bacteria were allowed to recover by incubating at 37°C in LB broth, plated out on to LB agar plates containing chloramphenicol (12.5 μ g/ml) and incubated at 37°C overnight. BAC plasmids were isolated from *E. coli* using an alkaline-lysis procedure and screened by PCR. Four sets of primers were used to amplify sequences in U_S6, U_L10, U_L30 and U_L40. Two micrograms of DNA from clones that were positive for all primer sets were transfected into a 100 mm dish of Vero cells using 18 μ l of lipofectamine and incubated at 34°C. Viral plaques were observed 2 days after transfection.

BAC mutagenesis

Bacteria (strain RR1) containing p25 (see text) were made electrocompetent using standard procedures.³² Forty microliters of electrocompetent cells were mixed with 2 μ l of pKO5-LARA in an ice-cold microfuge tube and transferred to a 0.1 cm electroporation cuvette (BioRad, Mississauga, ON, Canada) and electroporated at 1.8 kV with 25 μ farad and 200 ohm resistance. The bacteria were allowed to recover for 1 h at 30°C in 1 ml of LB, plated on zeocin/chloramphenicol LB agar plates and incubated overnight at 43°C. The following day, six colonies were picked and serially diluted into LB, plated on chloramphenicol/5% sucrose LB plates and incubated overnight at 30°C. To confirm loss of replacement vector, sucrose-resistant colonies were replica plated on to zeocin LB plates and incubated at 30°C. Potential positive colonies (sucrose/chloramphenicol resistant, zeocin sensitive) were picked directly into PCR buffer. Mutations were confirmed by PCR and Southern hybridization. Mutant vectors were tested for growth on 2–2 and Vero cells.

Amplicon production

2–2 Cells were cotransfected with 1, 2, or 4 μ g of either p45–25 (HSV-BAC Δ a₂) and pHSVlac¹¹ or with a *pac*-deleted HSV cosmid set and pHSVlac exactly as described by Fraefel *et al.*⁹ Cells were harvested after 4 days, frozen and thawed three times, the cellular debris removed by centrifugation and the supernatant used to infect fresh monolayers. Titers were calculated by serially diluting lysates and counting either plaques or transduced blue cells after staining with X-Gal as described by Fraefel *et al.*⁹ Transfections were repeated six times using the optimally determined DNA concentration to determine titer.

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References

- Gage PJ, Sauer B, Levine M, Glorioso JC. A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. *J Virol* 1992; **66**: 5509–5515.
- Qiu SH, Deshmane SL, Frazer NW. An *in vitro* ligation transfection system for inserting DNA sequences into the latency-associated transcripts (LATs) gene of herpes simplex virus type 1. *Gene Therapy* 1994; **1**: 300–306.
- Rixon FJ, McLaughlin J. Insertion of DNA sequences at a unique restriction enzyme site engineered for vector purposes into the genome of herpes simplex virus type 1. *J Gen Virol* 1990; **71**: 2931–2939.
- Roizman B, Jenkins FJ. Genetic engineering of novel genomes of large DNA viruses. *Science* 1985; **229**: 1208–1214.
- Cohen JI, Seidel KE. Generation of varicella-zoster virus (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication *in vitro*. *Proc Natl Acad Sci USA* 1993; **90**: 7376–7380.
- Cunningham C, Davison AJ. A cosmid-based system for constructing mutants of herpes simplex virus type 1. *Virology* 1993; **197**: 116–124.
- Kemble G, Duke G, Winter R, Spaete R. Defined large-scale alterations of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. *J Virol* 1996; **70**: 2044–2048.
- Tompkinson B *et al.* Epstein-Barr virus recombinants from overlapping cosmid fragments. *J Virol* 1993; **67**: 7298–7306.
- Fraefel C *et al.* Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. *J Virol* 1996; **70**: 7190–7197.
- Jacoby DR, Fraefel C, Breakefield XO. Hybrid vectors: a new generation of virus-based vectors designed to control the cellular fate of delivered genes. *Gene Therapy* 1997; **4**: 1281–1283.
- Fraefel C *et al.* Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. *Mol Med* 1997; **3**: 813–825.
- Stavropoulos TA, Strathdee CA. An enhanced packaging system for helper-dependent herpes simplex virus vectors. *J Virol* 1998; **72**: 7137–7143.
- Messlerle M *et al.* Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc Natl Acad Sci USA* 1997; **94**: 14759–14763.
- O'Connor M, Peifer M, Bender W. Construction of large DNA segments in *Escherichia coli*. *Science* 1989; **244**: 1307–1312.
- Quinn JP, McGeoch DJ. DNA sequence of the region in the genome of herpes simplex virus type 1 containing the genes for DNA polymerase and the major DNA binding protein. *Nucleic Acids Res* 1985; **13**: 8143–8163.
- Weller SK *et al.* Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. *Mol Cell Biol* 1985; **5**: 930–942.
- Gray CP, Kaerner HC. Sequence of the putative origin of replication in the UL region of herpes simplex virus type 1 ANG DNA. *J Gen Virol* 1984; **65**: 2109–2119.
- Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild type *Escherichia coli*: application to open reading frame characterization. *J Bact* 1997; **179**: 6228–6237.
- McGeoch DJ *et al.* The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol* 1988; **69**: 1531–1574.
- Garber DA, Beverley SM, Coen DM. Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. *Virology* 1993; **197**: 459–462.

- 21 Roizman B, Sears AE. Herpes simplex viruses and their replication. In: Fields BN, Knipe DM, Howley PM (eds). *Fundamental Virology*, 3rd edn. Lippincott-Raven: Philadelphia, 1996, pp. 1043–1107.
- 22 Moyal M *et al*. Heterogeneity of *Bam*HI DNA fragments B and E in several HSV-1 strains and recombinants. *Virus Genes*. 1988; **1**: 165–174.
- 23 Post LE, Conley AJ, Mocarski ES, Roizman B. Cloning of reiterated and nonreiterated herpes simplex virus type 1 sequences as *Bam*HI fragments. *Proc Natl Acad Sci USA* 1980; **77**: 4201–4205.
- 24 Igarashi K, Fawl R, Roller RJ, Roizman B. Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *J Virol* 1993; **67**: 2123–2132.
- 25 Sandri-Goldin RM, Mendoza GE. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. *Genes Dev* 1992; **6**: 848–863.
- 26 Horsburgh BC *et al*. Recurrent acyclovir-resistant herpes simplex in an immunocompromised patient: can strain differences compensate for loss of thymidine kinase in pathogenesis? *J Infect Dis* 1998; **178**: 618–625.
- 27 Geller AI. Influence of the helper virus on expression of beta-galactosidase from a defective HSV-1 vector, pHSVlac. *J Virol Meth* 1991; **31**: 229–238.
- 28 Horsburgh BC, Kollmus H, Hauser H, Coen DM. Translational recoding induced by G-rich mRNA sequences that form unusual structures. *Cell* 1996; **86**: 949–959.
- 29 Shizuya H *et al*. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F factor based vector. *Proc Natl Acad Sci USA* 1992; **89**: 8794–8797.
- 30 DeLuca NA, Schaffer PA. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. *Nucleic Acids Res* 1987; **15**: 4491–4511.
- 31 Chiou H, Weller SK, Coen DM. Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase inhibitors. *Virology* 1985; **145**: 213–226.
- 32 Seidman CE, Struhl K, Sheen J. *Short Protocols in Molecular Biology*. John Wiley and Sons: NY, USA, 1995.