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REVIEW

Cloning of herpesviral genomes as bacterial artificial chromosomes

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SUMMARY

Herpesviruses, which are important pathogens for both animals and humans, have large and complex genomes with a coding capacity for up to 225 open reading frames (ORFs). Due to the large genome size and the slow replication kinetics *in vitro* of some herpesviruses, mutagenesis of viral genes in the context of the viral genome by conventional recombination methods in cell culture has been difficult. Given that mutagenesis of viral genes is the basic strategy to investigate function, many of the herpesvirus ORFs could not be defined functionally. Recently, a completely new approach for the construction of herpesvirus mutants has been developed, based on cloning of the virus genome as a bacterial artificial chromosome (BAC) in *E. coli*. This technique allows the maintenance of viral genomes as a plasmid in *E. coli* and the reconstitution of viral progeny by transfection of the BAC plasmid into eukaryotic cells. Any genetic modification of the viral genome in *E. coli* using prokaryotic recombination proteins is possible, thereby allowing the generation of mutant viruses and facilitating the analysis of herpesvirus genomes cloned as infectious BACs. In this review, we describe the principle of cloning a viral genome as a BAC using murine gammaherpesvirus 68 (MHV-68), a mouse model for gammaherpesvirus infections, as an example.

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INTRODUCTION

Herpesviruses are important pathogens for both animals and humans [1]. After primary infection, they persist lifelong in their host in a latent state, interrupted by occasional episodes of reactivation. A prerequisite for establishing a persistent infection despite the host's immune system is the ability of herpesviruses to modulate the virus–host interaction [2–6]. Herpesviruses possess double-stranded DNA genomes of 100–250 kbp, containing up to 225 open reading frames (ORFs) [1]. These large and complex genomes can code for an extensive repertoire of gene functions required to interfere efficiently with the host immune response. Although the complete sequence of the genome of many herpesviruses has been determined, the precise function for many of the viral genes, both *in vitro*, and in particular *in vivo*, has not been elucidated. Knowing the function of individual or families of viral genes is important: (a) to understand their role in pathogenesis; (b) to examine their potential as a therapeutic target; (c) for the rational design of vaccines; and (d) for utilisation as a tool in gene therapy.

In principle, the strategy to assign a function to a viral gene in the context of the whole viral genome is mutagenesis of the gene of interest, followed by the analysis of the phenotype of the mutant virus both *in vitro* and *in vivo*. Conventional methods for mutagenesis of herpesviruses included chemical mutagenesis, site-directed mutagenesis by

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Abbreviations used
BAC, bacterial artificial chromosome; gfp, green fluorescent protein; gpt, guanosine phosphoribosyl transferase; HCMV, human cytomegalovirus; HHV-8, human herpesvirus 8; HVS, herpesvirus saimiri; KSHV, Kaposi's sarcoma herpesvirus; MCMV, mouse cytomegalovirus; MHV-68, murine gammaherpesvirus 68; PRV, pseudorabiesvirus; Tn, transposon.

homologous recombination in eukaryotic cells, and manipulating virus genomes using overlapping cosmid clones (reviewed, for example, in [7–9]). These methods proved very useful for the generation of a variety of mutants but their construction is often inefficient, laborious and time consuming, mainly due to the large genome size and, in case of the β- and γ-herpesviruses, the slow replication kinetics or the lack of replication *in vitro*. Conventional methods are performed in eukaryotic cells and therefore are difficult to control. One problem is that the analysis of the mutant viral genomes is only possible at the very end of the lengthy experimental procedure and thus additional changes in the viral genome such as deletions, rearrangements or illegitimate recombinations are frequently only observed then. Furthermore, the generation of a viral mutant requires selection against nonrecombinant wt virus and finally separation of the mutant virus from the wt virus, for example by plaque-purification. Additionally, complementing cell lines are required when mutations are introduced into essential viral genes. The construction of a mutant is therefore often a limiting step for the analysis of a viral gene function in the genomic context.

Recently, a completely new approach for the construction of herpesvirus mutants has been developed. This approach is based on cloning of the virus genome as a bacterial artificial chromosome (BAC) in *E. coli* [10]. Soon after the pioneering work of Messerle *et al.* [10], who cloned the entire genome of mouse cytomegalovirus (MCMV) as BAC, the cloning of several herpesviruses including human cytomegalovirus (HCMV), HSV, pseudorabiesvirus (PRV), EBV, Kaposi's sarcoma herpesvirus (KSHV, also called human herpesvirus 8 [HHV-8]) and murine gammaherpesvirus 68 (MHV-68) as BACs has been described [11–24]. This technique allows the maintenance of viral genomes as a BAC in *E. coli* and the reconstitution of viral progeny by transfection of the BAC plasmid into eukaryotic cells. Mutagenesis of the virus genome in *E. coli* using prokaryotic recombination functions is possible, thereby allowing the generation of mutant viruses. Using this method, any genetic modification should be possible, thereby facilitating the analysis of herpesvirus genomes cloned as infectious BACs. Here, we describe the principle of cloning a viral genome as BAC using MHV-68 as an example [18].

MHV-68 is a mouse model of γ-herpesvirus infection [25–32]. In humans, the prototypic γ1-herpesvirus, EBV, is associated with lymphomas and nasopharyngeal carcinoma [33], and HHV-8, a γ2-herpesvirus, is associated with Kaposi's sarcoma, primary effusion lymphomas and multicentric Castleman's disease [34]. *In vivo* studies of γ-herpesvirus pathogenesis have been limited to clinical investigation of the infection because of the restricted host range of these viruses. MHV-68 is a natural pathogen of wild murid rodents [35], and is capable of infecting laboratory mice. Clinically, MHV-68 infection of mice induces a syndrome very similar to EBV in humans [25]. Genetically, MHV-68 is similar to EBV, but more closely related to herpesvirus saimiri (HVS) and HHV-8 [36]. A major advantage of this model is the availability of genetically defined mouse strains rendered deficient for specific parameters, for example by gene-knockout technology. The genome of MHV-68 consists of 118 237 bp of unique sequence flanked by multiple copies of a 1213 bp terminal repeat, and contains genes common to other members of the gammaherpesviruses, homologues of cellular genes, but also genes specific for MHV-68 only [36]. The analysis of MHV-68 mutants will significantly contribute to the understanding of viral gene functions and to the evaluation of their role in the pathogenesis of gammaherpesvirus infections.

CONSTRUCTION OF A RECOMBINANT VIRUS CONTAINING THE BAC VECTOR SEQUENCES

For the generation of a virus BAC, the viral genome and the BAC vector have to be joined. Usually, the BAC vector is inserted into the virus genome by homologous recombination in eukaryotic cells which is comparable to the construction of a recombinant virus by the conventional mutagenesis technique (Figure 1). This could in theory also be achieved by ligation of the linear viral genome to the BAC vector *in vitro*. In the case of MHV-68, the left end of the genome was chosen for the integration of the BAC vector sequences since this region has been shown to be dispensable for lytic replication *in vitro* and for latent infection *in vivo* [37]. Additionally, that study and previous work describing the generation of HVS recombinants showed that in the case of gammaherpesviruses, insertions between the unique region of the DNA

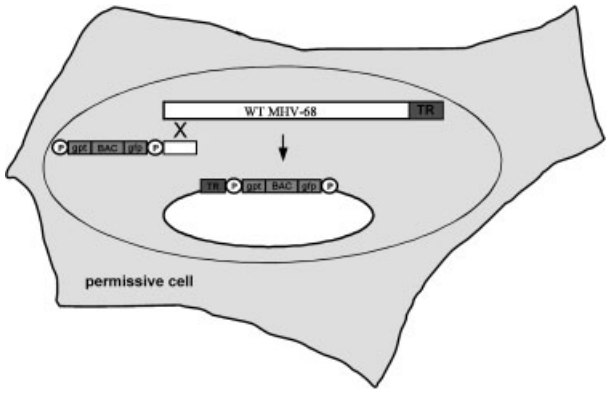

Figure 1. Construction of the MHV-68 BAC genome. The BAC-cloned genome was generated in eukaryotic cells by homologous recombination of MHV-68 DNA with a recombination plasmid containing 1.5 kbp of flanking homologous sequence (white box) as well as the BAC vector (BAC), the guanosine phosphoribosyl transferase gene (*gpt*) and the gene for the green fluorescent protein (*gfp*) flanked by loxP sites (P). Viral DNA and the linearised recombination plasmid were co-transfected into eukaryotic cells to generate a recombinant virus. Circular DNA of the recombinant virus genome was then isolated from cells [58] and electroporated into *E. coli*. TR: terminal repeats

and the terminal repeats can be achieved [37,38]. Whereas for other herpesviruses the experimental recombination is usually based on two homology regions, here, a single crossing-over event via only one homologous region at one side of the recombination plasmid suffices. Thus, to insert the BAC vector sequences into the genome of MHV-68, a recombination plasmid was constructed containing a 1.5 kbp fragment homologous to the left end of the unique region of the MHV-68 genome (the desired integration site) and the BAC vector including the genes for *gpt* (guanosine phosphoribosyl transferase) and *gfp* (green fluorescent protein) which served as selection and screening markers, respectively (Figure 1). After cotransfection of both recombination plasmid and MHV-68 DNA into permissive fibroblasts, the development of virus plaques showing green fluorescence indicated the integration and expression of the *gfp* gene. Recombinant viruses were selected utilising the *gpt* marker in the presence of mycophenolic acid and xanthine [39]. The *E. coli* enzyme *gpt* is able to synthesise and to provide purine precursors from xanthine in order to support replication of recombinant viruses under conditions where cellular purine synthesis is blocked by mycophenolic acid.

TRANSFER OF THE RECOMBINANT GENOME FROM INFECTED CELLS TO *E. COLI*

Since the linear, double-stranded DNA genome of herpesviruses circularises during replication, circular viral DNA can be isolated from infected cells. In the case of MHV-68, this was done after three rounds of selection. The circular replication intermediate was then transferred to *E. coli* by DNA transformation. It is important to note that this transfer into *E. coli* is needed only once. Isolation of plasmids from single *E. coli* colonies and restriction enzyme analysis led to the identification of a bacterial clone containing a BAC plasmid with the full-length MHV-68 genome.

PROPAGATION AND MUTAGENESIS IN *E. COLI*

Once the BAC is transferred to *E. coli*, it can be maintained, propagated and mutated. Only at the very end of all manipulations is the BAC DNA, which is clonal, transferred by transfection into permissive cells to reconstitute virus. Mutagenesis of the large BAC in *E. coli* is achieved using the bacterial recombination machinery. Conventional cloning techniques are not applicable due to the large size of the genome. In principle, mutagenesis can either be targeted or random. For targeted mutagenesis, a two-step replacement procedure [10,40] or a one-step method [18,41] have been applied. Random mutagenesis was achieved by transposon insertion [42].

Targeted mutagenesis—two-step replacement procedure

In principle, a shuttle plasmid has to be constructed containing the mutant allele flanked by regions homologous to the desired integration site of the mutation in the BAC plasmid. In the first step, the shuttle plasmid is inserted into the BAC plasmid by homologous recombination, leading to a so-called cointegrate (Figure 2). The homologous recombination is mediated by the bacterial protein RecA, either expressed by the bacterial strain used, or provided with the shuttle plasmid. In the second step, the cointegrate may spontaneously resolve by homologous recombination to either wildtype or mutant BAC plasmid. As the resolution of the cointegrate is not a frequent event, selection against bacteria containing non-resolved cointegrates using negative selection

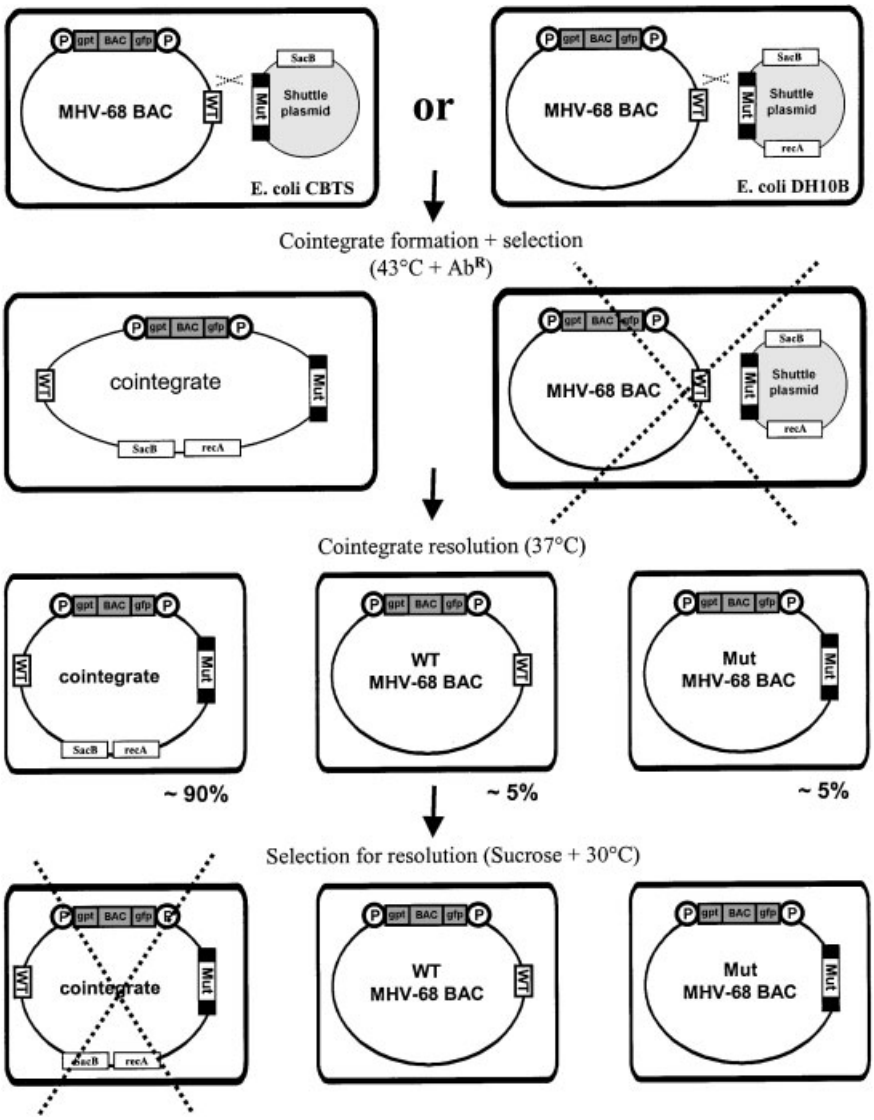

Figure 2. Two step replacement procedure (shuttle mutagenesis) for mutagenesis of the BAC cloned MHV-68 genome in *E. coli*. The mutagenesis can be performed in recombination-proficient *E. coli* strains such as CBTS (upper left panel) or in recombination-deficient *E. coli* strains such as DH10B (upper right panel). The mutant allele (Mut), flanked by sequences homologous to the desired integration site, is provided by a shuttle plasmid with a temperature-sensitive replication mode and the negative selection marker SacB which allows bacteria harbouring a resolved cointegrate to be selected. The shuttle plasmid also contains a gene to confer antibiotic resistance (not shown) while the BAC contains a resistance gene for a second antibiotic (not shown). For mutagenesis in recombination-deficient *E. coli*, the shuttle plasmid also has to provide the *rec*A gene. In a first step, the shuttle plasmid carrying the desired mutation plus flanking homologies (black boxes) is transformed into bacteria already containing the BAC plasmid. Through homologous recombination via one of the two homologies, the shuttle plasmid is completely integrated into the viral BAC genome, leading to a cointegrate. Bacteria containing the cointegrate are selected by incubation at 43°C plus both antibiotics. Resolution of the cointegrate via one of the two homologies leads to either the wildtype (WT) or the mutant (Mut) BAC plasmid. Bacteria harbouring a resolved cointegrate are selected at 30°C on agar plates containing 5% sucrose (counterselection against SacB) together with the antibiotic whose resistance gene is encoded on the BAC

markers (which have been integrated into the shuttle plasmid, for example, the *sacB* gene) is to be preferred. In a last step, bacterial clones harbouring the mutant or the parental BAC have to be identified (see below). Various modifications of the two-step replacement procedure have been developed in the meantime [14,40,43]. The major advantage of the two-step replacement method is that no traces of foreign sequences such as selection markers etc. remain in the BAC genome after

mutagenesis. However, this method is more laborious and time consuming than other methods described below, since it requires construction of a shuttle plasmid via multiple cloning steps. Easier and more powerful procedures have therefore been developed recently.

Targeted mutagenesis—one-step method

Recently, a one-step mutagenesis method named 'ET cloning' has been developed [41,44]. This method uses the recombination functions *recE* and *recT* or *redα* and *redβ* for introduction of mutations into a circular DNA molecule. A linear DNA fragment containing a selectable marker and short homologous sequences flanking the target sequence is transferred into recombination proficient *E. coli*. The selectable marker along with the mutation is then introduced by a double crossing-over event (Figure 3). To generate mutants of MHV-68, linear PCR fragments containing a FRT (FLP recognition target sites)-flanked tetracycline resistance cassette [45] were generated using primer pairs that contained 24 nucleotides specific for the tetracycline resistance gene, and an additional 50 nucleotides homologous to sequences flanking the region to be targeted. The PCR products can then be transferred into the MHV-68 BAC by homologous recombination in *E. coli* strain JC8679 [46] already containing the MHV-68 BAC and constitutively expressing *recE* and *recT*, or in *E. coli* strain DH10B already containing the MHV-68 BAC and a plasmid encoding the lambda recombination proteins *redα*, *redβ* and *redγ* [44,47]. By flanking the tetracycline resistance gene with FRT sites, the selection marker can be removed by recombinase Flp-mediated recombination [45].

There are several advantages of the ET mutagenesis protocol, when compared with the two-step replacement procedure. First, it does not require construction of a recombination plasmid via multiple cloning steps, and is therefore a very fast procedure which is not dependent on the suitable disposition of restriction enzyme cleavage sites. Second, because of the short homology required, the complete modified region can be sequenced in a single step to determine whether recombination had occurred as planned. This method in particular may be useful for mutagenesis of BAC-cloned virus genomes for which only limited sequence information is available. Knowledge of short sequences (50 bp) suffices for the generation

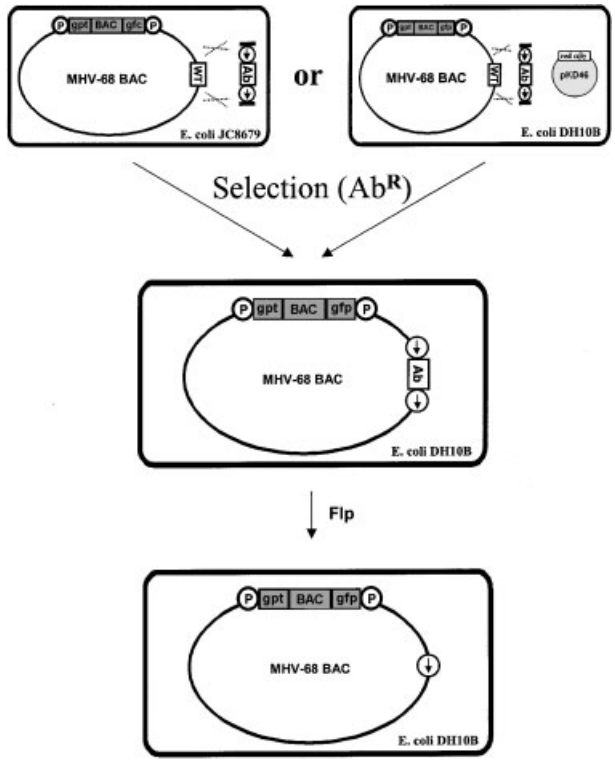

Figure 3. One step replacement procedure (ET cloning) for mutagenesis of the BAC cloned MHV-68 genome in *E. coli*. The procedure can be performed in *E. coli* strains such as JC8679 [46] which constitutively express recE and recT or in recombination-deficient *E. coli* strains such as DH10B. In the latter case, a plasmid (for example, pKD46 [44]) expresses the red recombination functions under the control of a regulated (L-arabinose-inducible) promoter. A selectable marker gene (for example, an antibiotic resistance gene [Ab]), flanked by FRT sites (⊖), is created by PCR using primers which contain homologies of about 50 nucleotides according to the desired integration site at their 5′-ends, the mutation and priming regions specific for the selectable marker gene. The generated linear DNA fragment is transferred to the MHV-68 BAC by a double crossing-over event. The selectable marker gene can finally be excised by recombinase FLP, leaving one FRT site behind

of the recombination fragment. On the other hand, there are also disadvantages. One is the potential instability of the BAC plasmid since short repeats in the herpesviral genome can lead to unwanted recombination in recombination competent bacteria (see below). Another is the need to insert a selectable marker. Although the marker can be removed using flanking FRT or similar sites, usually foreign sequence traces will remain in the BAC plasmid which may interfere with gene expression. Nevertheless, ET mutagenesis is the method of choice for introduction of deletions or (short) insertions into BAC-cloned virus genomes.

Random mutagenesis: transposon insertion

Another possibility for mutagenesis of BAC plasmids is direct transposon (Tn) mutagenesis [42]. Transposons are mobile genetic elements that can randomly insert into DNA. Thus, transfer of a Tn donor plasmid into *E. coli* already containing the BAC plasmid leads to random insertion of the Tn into the viral BAC plasmid (Figure 4). Afterwards, the temperature-sensitive Tn donor plasmid is eliminated at a temperature not permissive for plasmid propagation. The Tn insertion site can directly be determined by sequencing from primer sites within the Tn. This is of advantage when compared with the previous random mutagenesis procedures like chemical mutagenesis. Selected mutants can be identified by PCR screening procedures [42,48]. The power of the method lies in the possibility of generating large libraries of mutant BAC genomes with random insertion of the transposon and screening the libraries for mutants displaying specific phenotypes [48–50].

ANALYSIS OF MUTATED BAC PLASMIDS

BAC plasmids can be isolated from *E. coli* cultures using standard procedures like alkaline lysis [51], and analysed by restriction enzyme digestion and gel electrophoresis (Figure 5). Further analysis is usually done by Southern blot analysis and/or by sequencing of the targeted region. An advantage of this technology, when compared with conventional mutagenesis in cell culture, is increased safety. Since the viral genome is kept as a BAC throughout all steps of manipulation, there is no need to handle infectious viruses. In addition, the viral sequences at the time of cloning are preserved, since *in vitro* culture of the virus is not necessary. This is in contrast to the conventional construction of viral mutants in cell culture, where the *in vitro* culture may already result in changes to the virus. This is most relevant for the analysis of fresh clinical isolates.

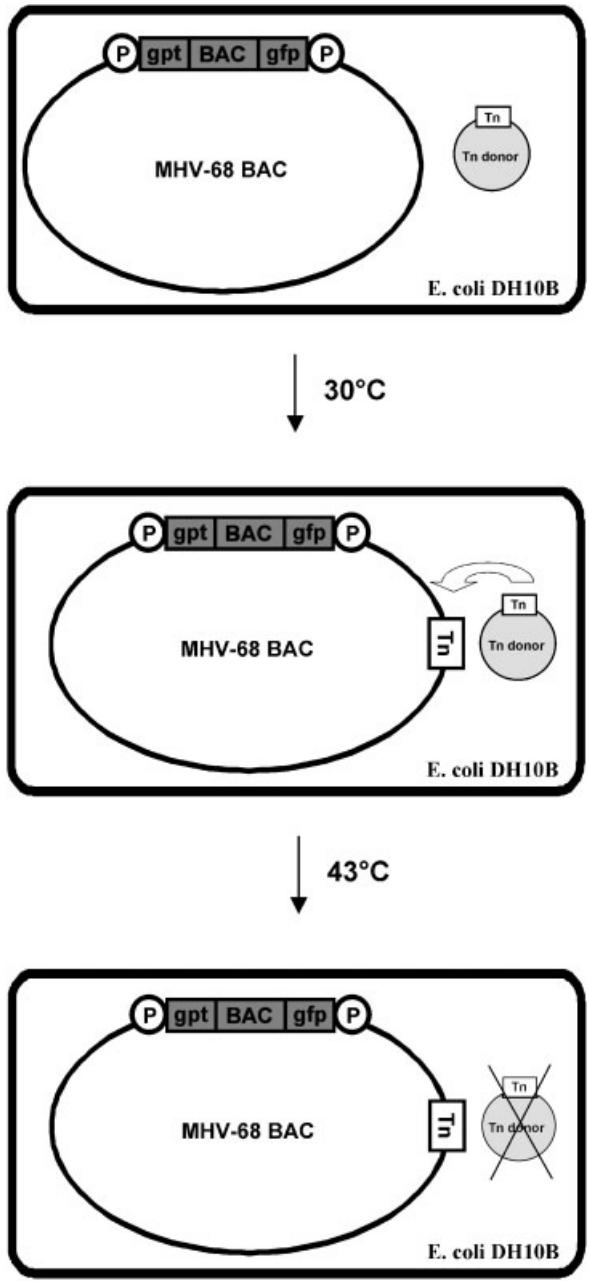

Figure 4. Transposon mutagenesis of the BAC cloned MHV-68 genome in *E. coli*. A transposon (Tn) donor plasmid (Tn donor) with a temperature-sensitive replication mode is introduced into *E. coli* carrying the MHV-68 BAC. At the permissive temperature, the Tn inserts into the BAC. Appropriate selection at the nonpermissive temperature removes the Tn donor plasmid and selects for BAC plasmids with a Tn insertion

RECONSTITUTION OF INFECTIOUS VIRUSES, ANALYSIS OF VIRAL GENOMES, AND REMOVAL OF BAC VECTOR SEQUENCES

In general, virus reconstitution is achieved by transfection of the viral BAC plasmid into permissive cells either by electroporation or by chemical methods (Figure 6). Another approach is the use of invasive bacteria to transfer the viral BAC plasmid into eukaryotic cells [49]. This transfer makes use of *E. coli* which can invade permissive cells due to the expression of the bacterial genes invasin

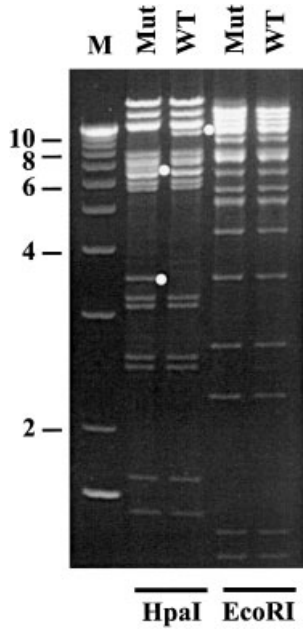

Figure 5. Analysis of BAC plasmids. For example, a subtle mutation, generating a new HpaI site, was introduced into the MHV-68 BAC. Mutant (Mut) and wildtype (WT) BAC plasmids were isolated from *E. coli* cultures, and digested with HpaI and EcoRI, respectively. Then, DNA fragments were separated by agarose gel electrophoresis. As expected, Mut or WT BAC plasmids can be differentiated by HpaI, but not by EcoRI digestion, according to the DNA fragments which are characteristic for the Mut or WT, respectively (indicated by dots). The EcoRI digestion was done to further confirm the integrity of the two plasmids

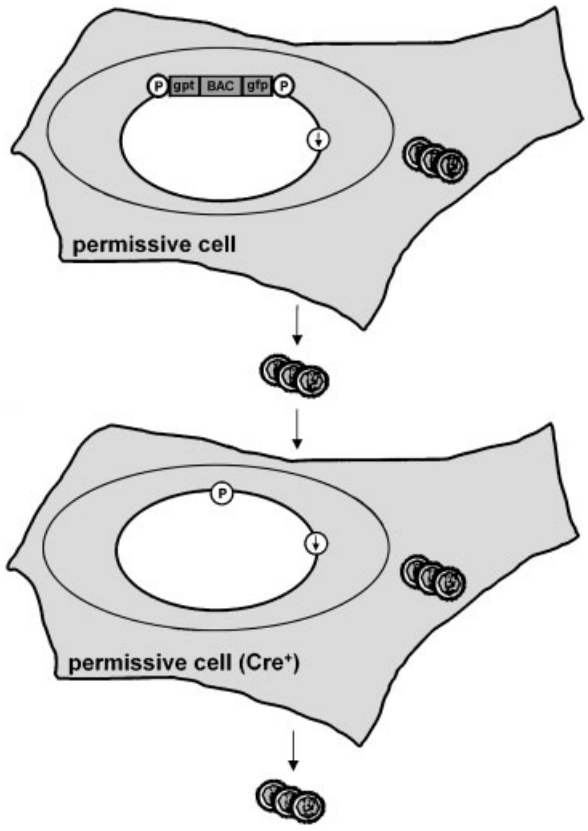

Figure 6. Reconstitution of infectious viruses. The mutated MHV-68 BAC plasmid is transfected into permissive eukaryotic cells to reconstitute recombinant virus. Propagation of the mutant virus in fibroblasts expressing recombinase Cre results in deletion of the BAC vector sequences. ⊖ : FRT-site. P: loxP-site

and listeriolysin. If permissive cells are available in which the particular reconstituted virus undergoes lytic replication and causes cpe, for example in fibroblasts, virus reconstitution can be monitored easily by the appearance of cpe. For example, transfection of the MHV-68 BAC plasmid into BHK-21 or NIH3T3 cells leads to the development of plaques. For herpesviruses which show no lytic replication *in vitro*, such as EBV and HHV-8, expression of marker genes like gfp has been used to monitor reconstitution and transmission of infectious virus [11,21,23]. The structure of the genomes of the reconstituted viruses are determined by analysing DNA isolated from infected cells. Basically, the same methods as described above for the BAC plasmids are applied, including restriction enzyme digestion and gel electrophoresis as well as Southern blot analysis and/or sequencing of the targeted region.

As described above, cloning of a viral genome as a BAC requires the insertion of the BAC vector sequences into the viral genome. This raises the question of whether the presence of the BAC vector sequences in the BAC-derived viruses may interfere with the biological properties of these viruses *in vitro* and, more importantly, *in vivo*. We could indeed show that for *in vivo* studies with MHV-68 BAC-derived viruses, the BAC vector sequences have effects *in vivo* and should be removed [52]. Observations that emphasise the need to remove BAC vector sequences from the viral genome have been made with other BAC-cloned herpesviruses including MCMV, HCMV and PRV as well [17,22,53]. Thus, we and others constructed the BAC-cloned genome in a way that vector sequences can be deleted [17,22,52,53]. For MHV-68, the BAC vector sequences, including the genes for *gpt* and *gfp*, were flanked by loxP sites. By propagation of

MHV-68 BAC-derived virus in cells expressing recombinase Cre, viruses devoid of BAC vector sequences can be isolated (Figure 6). For MCMV, the BAC vector sequences were flanked by identical viral sequences. Complete excision of the BAC vector from the viral population required several rounds of replication [53]. For PRV and HCMV, excision of the BAC vector sequences was achieved by Cre recombinase which was encoded by the BAC vector itself. Viruses lacking the BAC vector sequences could thus be isolated from transfected cells without the need for serial passage or plaque purification [17,22]. In contrast to MCMV, HCMV and PRV, where the BAC vector sequences are excised whenever the BAC-cloned genomes are delivered into mammalian cells, the BAC vector sequences in the BAC-derived MHV-68 can be removed at will. Since the sequence for gfp is included in the MHV-68 BAC-cassette, recombinant viruses with or without gfp expression can be generated, depending on the needs of the particular experiment. Whereas in MCMV the resulting virus is completely free of bacterial sequences, in HCMV, PRV and MHV-68, a single 34 bp loxP site remains in the viral genome after excision of the BAC vector sequences [17,22,52,53]. Therefore, the procedure to remove BAC sequences in the genome is a matter of choice dictated by the intended experiments.

STABILITY OF VIRAL GENOMES CLONED AS BAC PLASMIDS

An important aspect of cloning a viral genome as a BAC is the stability of the BAC plasmids during maintenance, propagation and mutagenesis in *E. coli*. Since viral genomes often contain direct repeats that are prone to undergo recombination [54], there is a risk of obtaining deletions or rearrangements if the BAC plasmid is maintained in recombination-proficient bacteria. Therefore, the BAC plasmids are usually kept in recA-negative bacterial strains like DH10B, although there is also recA-independent recombinogenic activity. On the other hand, recombination functions are needed for mutagenesis of the genomes. These functions can be provided by (i) transfer of the BAC plasmid to a bacterial strain which is either constitutively or conditionally expressing recombinogenic functions like recA [10,14,55], or (ii) transient expression of recombination functions like recA or recE and recT from either suicide plasmids or plasmids with inducible promotors [41,44]. In any case, it has to be determined how much recombinogenic activity is needed for successful mutagenesis without risking rearrangements of the particular viral genome under study. In general, it appears that the viral BAC plasmids do not undergo recombination or deletion when they are maintained in *E. coli* under appropriate conditions [7,8]. Meanwhile, it has been shown that different BAC-derived viruses displayed biological properties *in vivo* comparable to wt virus, a strong indication for maintenance of genome integrity in *E. coli* [14,52,53]. However, these conditions have to be defined and may be different for different viral BACs.

For the MHV-68 BAC genome, stability was tested by propagation in the *E. coli* strain DH10B. This strain carries a mutation in the *rec*A gene and is therefore severely impaired in its ability to perform homologous recombination. The MHV-68 genome contains a number of repeats, i.e. the terminal repeats, an internal 100 bp repeat and an internal 40 bp repeat [36] which may be prone to recombination events. Recombination between direct repeats causes the deletion of intervening sequences and a reduction in the copy number of the repeated sequences. Changes were only observed in the repetitive sequences of MHV-68, but not in other regions of the MHV-68 BAC plasmid [18]. Most frequently, the loss of some of the 40 bp repeat units was observed, whereas changes within the fragments containing the 100 bp internal repeat or the terminal repeat seemed to be very rare and were only observed sporadically. Size variations due to alterations in repeat structures also occur naturally in other gammaherpesviruses [56]. Whether the number of repeats in the MHV-68 WT genome remains constant under physiological conditions is not known [57]. The loss of some of the 40 bp repeats had no influence on the reconstitution of infectious virus and on the *in vitro* growth properties [18]. Experiments are underway to determine whether the variability of the MHV-68 repeat structures has any biological effect *in vivo*.

CONCLUSIONS

Several herpesvirus genomes have now been cloned as BACs in *E. coli*, and the cloning of additional herpesvirus genomes is ongoing. Efficient site-specific mutagenesis procedures for the

cloned BAC plasmids as well as methods for random mutagenesis and screening procedures for libraries of randomly generated mutants have been established by a growing number of laboratories. Clearly, the BAC technology represents a milestone in the genetic analysis of herpesviruses with many promising applications.

For MHV-68, we have shown that the BAC-cloned genome is suitable for the construction of recombinant viruses and that the phenotypes can be reliably assessed *in vivo*. The BAC-technology will considerably speed up the construction of mutants in order to assess the role of viral genes in the virus–host interaction. Thus, this combination of techniques and methods will significantly contribute to the potential of MHV-68 infection of mice as a small animal model for gammaherpesvirus infections.

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