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# Bacterial Genetics of Large Mammalian DNA Viruses: Bacterial Artificial Chromosomes as a Prerequisite for Efficiently Studying Viral DNA Replication and Functions

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## 1. Introduction

Large DNA viruses such as herpesviruses and poxviruses constitute a group of highly relevant pathogens for animals and humans. Genetic and functional analysis of these viruses has been a constant research challenge mainly because of their large and complex genomes, which are difficult to access by standard molecular biology techniques. Homologous recombination is the major principle for the manipulation of such viral genomes. The simple site-directed integration of a selection marker gene into the viral DNA allowed for the first time the enrichment of recombinants in permissive eukaryotic cells (Mocarski et al., 1980; Smiley, 1980). Essential genomic sites including those involved in DNA replication cannot be efficiently investigated by this method, since mutants with such defects would not start to replicate and, thus, would not be generated. To some extent, amplicon constructs in the presence of a wild-type helper virus have been instrumental for functional studies, which were, however, hampered by the background of similar helper virus sequences. Such limitations were resolved by cloning entire virus genomes as cosmid libraries in *Escherichia (E.) coli* (van Zijl et al., 1988). The virus is reconstituted in permissive mammalian cells after cotransfection of overlapping viral cosmids. Essential genome sites involved in DNA replication can then eventually be identified by providing the missing function through *trans*-complementation. However, unwanted second-site mutations after recombination of the homologous overlapping sequences and the resulting spontaneous mutation possibilities at the overlapping stretches are considerable disadvantages, rendering this procedure rather unreliable.

The major methodological break-through was achieved by cloning entire herpesvirus genomes as infectious large plasmids, co-called bacterial artificial chromosomes (BACs) in *E. coli* (Messerle et al., 1997). This technology was first introduced for the murine cytomegalovirus genome and was subsequently applied to numerous other virus pathogens. This permitted the stable maintenance and the targeted mutagenesis of the virus genomes as single plasmids in *E. coli*. Afterwards, the transfer of the BAC DNA into virus-permissive eukaryotic cells allowed the reconstitution of the mutant virus representing a homogenous population. Initially, the selection marker and the BAC cassette remained obligatory after the manipulation of the genomes in *E. coli* and the virus reconstitution in eukaryotic cells, respectively. As an improvement, site-specific recombinase systems were applied for the excision of the BAC vector sequences, leaving behind only one recombinase recognition site. Also transposon mutagenesis was used for generating libraries of virus genomes saturated with single-site mutations. As the second break-through, manipulation techniques such as *en passant* mutagenesis enabled the seamless alteration of the BAC DNA in bacteria and, thus, the reconstitution of mutant progeny virus which was free of secondary mutations such as selection markers or recombinase recognition sites (Tischer et al., 2006). The third break-through was achieved, when all BAC vector sequences were deleted autonomically after transfection of restructured BACs into permissive eukaryotic cells, allowing the generation of virus progeny completely devoid of any operational sequences. This approach uses functional features of virus DNA replication in order to reconstitute the wild-type configuration at the previous BAC vector insertion site. In addition, methods were developed for the targeted transposition of the vector sequence within a BAC construct in order to optimize genomic vector design. Thus, BAC constructs of large mammalian DNA viruses have become crucial for functional studies, even of essential genes including that for viral DNA replication.

In this review, we summarize the development of viral BAC vectors and the bacterial genetics tools used. We discuss the advantages and disadvantages of different BAC vector strategies for herpes- and poxviruses, as well as the application potential for functional studies into DNA replication and other viral functions and the perspectives for future preventive and therapeutic strategies.

## 2. Generation of recombinant large DNA viruses

Herpesvirus genomes consist of 120-250 kb double-stranded (ds) linear DNA which is circularized after infection of the cell. They are composed of different unique and repetitive regions with densely packed or even overlapping open reading frames of approximately 70-220 genes. Poxvirus genomes are even larger. Whereas poxviruses replicate in the cytoplasm, the DNA replication of herpesviruses occurs in the nucleus. Functional studies in large DNA viruses require the precise generation of virus mutants; thus, accessory operational sequences and selection markers should be avoided in order to exclude unwanted side-effects which may hide or distort the effects mediated by the precise mutant position. The development of precise mutagenesis protocols has been a constant challenge in herpes- and poxvirology. Eight human herpesviruses are of major interest in this research field: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (hCMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and Kaposi's sarcoma-associated herpesvirus (KSHV). In addition, a series of animal herpesviruses have been subjected to recombinant mutagenesis. While all these herpesviruses are capable of persisting life-long in the human

body, they can be reactivated and cause disease under specific conditions during primary infection or during reactivation. Among the poxviruses, the major interest lies in vaccinia viruses which are highly important tools for vaccine development.

## 2.1 Basic strategies for the manipulation of large viral genomes

The classical, pre-recombinant strategy for mutant generation was developed approximately forty years ago by the targeted phenotypic selection of chemically induced and randomly generated temperature-sensitive variants which are conditionally expressing specific virus proteins (Schaffer, 1975). This strategy was a pacemaker for virus research and allowed the temperature-dependent study of defined virus functions, including properties which are essential for virus replication. However, the precise definition of the respective mutation was demanding and unwanted second-site mutations were difficult to exclude.

The size of herpes- and poxviral genomes was by far too large in order to utilize naturally occurring unique endonuclease recognition sites for the precise manipulation of the viral DNA. The targeted mutagenesis of herpes- or poxviral genomes was successfully achieved by the integration of a selection marker flanked by viral DNA sequences including the desired genetic mutation by homologous recombination into the virus genome during virus replication in cultured permissive eukaryotic cells (Manning & Mocarski, 1988; Mocarski et al., 1980; Post & Roizman, 1981; Smiley, 1980; Spaete & Mocarski, 1987). Since the homologous recombination occurred as a rare event, the recombinants were difficult to isolate from a dominating amount of wild-type virus. This method did not yet allow the targeting of essential genes, since the strategy was dependent on active virus replication.

A considerable step forward was done by introducing the concept of virus reconstitution by cotransfection of overlapping genomic cosmid clones into permissive cells (Cohen & Seidel, 1993; Cunningham & Davison, 1993; Kemble et al., 1996; Tomkinson et al., 1993; van Zijl et al., 1988). For this purpose, virion DNA was prepared, degraded to fragment sizes of approximately 30-40 kb, and cloned in *E. coli* into cosmid vector libraries. Selected overlapping and complementing genomic cosmid DNA clones were then selected and transfected as sets of three to five cosmids into permissive cells in order to reconstitute infectious virus. The major advantage of the cosmid complementation method is the fact, that the cloned viral DNA can be manipulated precisely by molecular biology or bacterial genetic techniques. Moreover, the obtained virus progeny is free of cosmid vector sequences, although selection markers cannot be avoided in most cases. By providing essential functions *in trans* or by *trans*-complementing cell lines, even essential virus genes were analyzed using this method. The cosmid-complementation technique is limited in eukaryotic cells by illegitimate events of homologous recombination or unwanted second-site mutations which are difficult to exclude. The technique of cosmid complementation was instrumental for developing entire virus genomes in the form of viral BACs.

## 2.2 Bacterial artificial chromosomes of entire large viral genomes

BACs were established for studies in human and animal genetics. BACs are single-copy bacterial F-factor-derived plasmids of approximately 7.5 kb which carry an own origin of replication, encode own DNA replication factors (e.g., repE), and an antibiotic resistance function (e.g., against chloramphenicol). BACs can stably maintain DNA molecules of up to 300 kb in recombination-deficient *E. coli* strains (Shizuya et al., 1992). A frequently used BAC vector is pBeloBAC11. The F-plasmid based vector sequences are often designated "mini-F"

fragments. The BAC copy number is strictly restricted to one or two copies per bacterial cell by regulatory elements (*e.g.*, *parA* & *parB*) of the mini-F vector. Thus, intermolecular homologous recombination events are largely excluded. However, repeated or duplicated sequences can still undergo homologous recombination (Shizuya et al., 1992). Bacteriophage P1-based vectors (PACs) are comparable to BACs. In contrast to BACs, yeast artificial chromosomes (YACs) can accommodate even larger inserts. However, YACs often have chimeric structures and sequence rearrangements (Ramsay, 1994; Schalkwyk et al., 1995). BACs show higher insert stability in *E. coli* in comparison to cosmid-based plasmids, which are restricted to DNA-fragment sizes of up to 50 kb (Kim et al., 1992). Therefore, BACs have become the vectors of choice for the cloning of large and complex genomes in *E. coli* (Ioannou et al., 1994; Shizuya et al., 1992). Moreover, BACs are instrumental in sequencing strategies, for functional genomics, and for the construction of gene targeting or gene therapy vectors (Copeland et al., 2001; Sparwasser & Eberl, 2007; Yang & Gong, 2005). For the application in cloning large DNA virus genomes, the BAC vector needs flanking virus homology regions of 300-500 bp for the precise targeting into the desired genomic region. Depending on the planned applications, accessory operational markers are included, such as genes for an autofluorescent protein, luciferase, or antibiotic resistance. Many strategies include flanking recognition sites (*e.g.*, *loxP*) for recombinases (*e.g.*, Cre) at the ends of the BAC insert sequence in order to allow the vector excision from recombinant progeny viruses.

Herpes- and also poxvirus BACs (Figure 1) can be constructed by inserting a mini-F vector into a specific site of a non-essential genomic region via homologous recombination of a linearized recombination construct during active virus replication in permissive eukaryotic cells after lipofection or electroporation. The recombination fragment may be cotransfected with virion DNA or the transfected culture may be superinfected with wild-type virus after transfection. The transfer of the circular replication intermediates of recombinant progeny viruses or artificially created circular DNA is carried out into a *RecA*<sup>-</sup> *E. coli* strain, such as DH10B (Messerle et al., 1997). Alternatively, the BAC vector can be inserted into a non-essential region of a specific viral cosmid clone, in addition to the pre-existing cosmid vector fragment. After cotransfection of sets of three to five overlapping viral cosmids into permissive cells, the BAC vector-containing virus is reconstituted. Similarly, the circular replication intermediates are then transferred into *E. coli* (Saeki et al., 1998; Tischer et al., 2007). A detailed molecular analysis of the viral BACs is necessary in order to show genetic integrity. Importantly, viral BAC DNA can be prepared in large quantity and high quality in order to compensate for the inefficient transfection procedures into permissive eukaryotic cells. After retransfer of the viral BAC into permissive cells, the resulting recombinant virus is reconstituted and compared with wild-type virus for genome structure and replication properties. If possible, plaque purification is recommended in order to ensure a homogenous virus population. Selectable markers such as genes for autofluorescent proteins may be useful for the rapid identification of recombinant viruses. A detailed analysis is necessary since unwanted genotypic and phenotypic changes in viral BACs have been observed (Ali et al., 2009; Messerle et al., 1997). In viruses with particular large genomes, such as cytomegaloviruses, the 7.5 kb BAC vector insert may already lead to obvious retardation of viral replication (Yu et al., 2002).

After the initial cloning of murine cytomegalovirus (mCMV; Messerle et al., 1997), the genomes of many herpesviruses, some poxviruses, and some large RNA viruses were constructed as infectious viral BACs (Table 1; Adler et al., 2003; Britt, 2000; Brune et al., 1999,



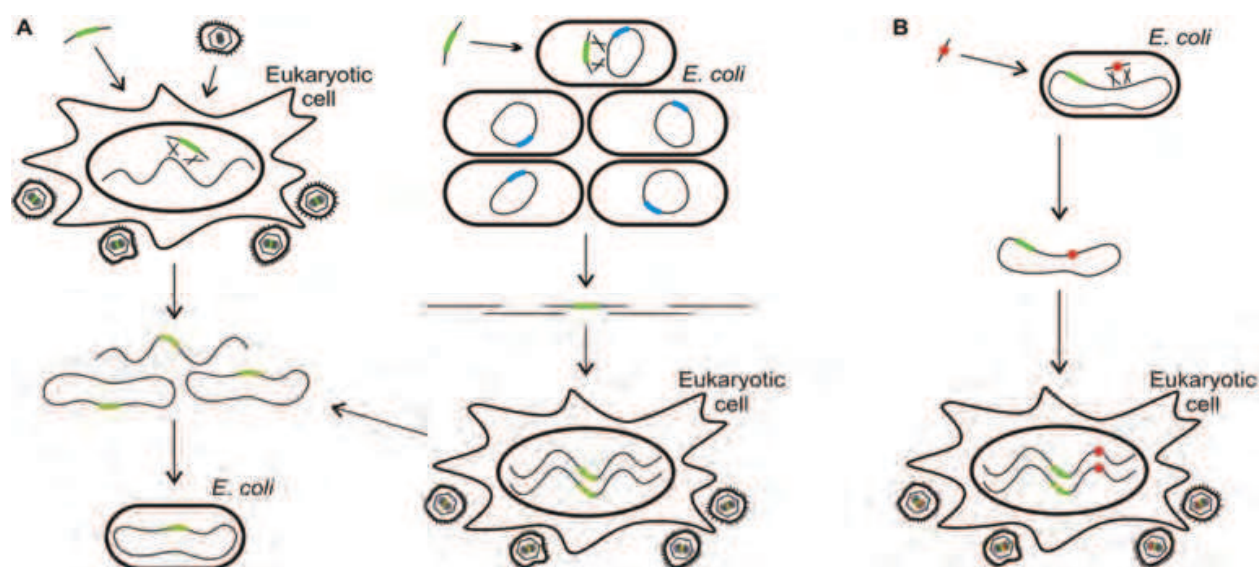


Fig. 1. Generation and mutagenesis of herpesvirus BACs. A) Cloning of a herpesviral genome as a bacterial artificial chromosome (BAC). A mini-F vector (green) is inserted into the viral DNA via homologous recombination (crossed lines) during virus replication in infected permissive eukaryotic cells. Circular replication intermediates are isolated and transferred into *E. coli* to establish an infectious viral BAC. Alternatively, the mini-F plasmid is first inserted into a viral cosmid (blue) DNA clone. Overlapping linear viral genome fragments from cosmid vectors are subsequently transfected into permissive cells. Circular DNA intermediates of recovered replicating virus are then isolated and transformed into *E. coli*. B) Herpesvirus BAC technology. The herpesviral BAC is maintained in *E. coli* and a mutation is introduced into the viral DNA by homologous recombination. The BAC is isolated and transfected into permissive eukaryotic cells, where mutant progeny is reconstituted (adapted from Felix Wussow, Ph.D. thesis, Christian Albrecht University of Kiel, 2009).

2000; Feederle et al., 2010; McGregor & Schleiss, 2001b; Wagner & Koszinowski, 2004; Wagner et al., 2002, 2004; Warden et al., 2011). Full-length viral DNA can be maintained and mutagenized in *E. coli* and delivered into permissive eukaryotic cells for virus reconstitution. Since poxviruses replicate in the cytoplasm, the initiation of viral transcription and DNA replication requires the presence of a related helper virus (Domi and Moss, 2002). Infectious homogenous progeny is recovered from mutated viral BAC-DNA in a defined manner without any further homologous recombination events to restore genome integrity, in contrast to virus reconstitution from overlapping cosmid fragments. Nevertheless, mini-F sequences in a non-essential genomic region can interfere with specific viral functions especially in further in vivo experiments. The complete removal of the mini-F vector by homologous recombination has been limited to laborious cotransfection experiments or to BAC constructs with restricted stability in bacteria (Strive et al., 2007; Wagner et al., 1999; Yu et al., 2002). Alternatively, the vector elements can be flanked by recognition sites for site-specific recombinases, which leave behind only one small recognition sequence (Adler et al., 2000, 2001; Chang & Barry, 2003; Smith & Enquist, 2000; Strive et al., 2006; Tanaka et al., 2003; Zhao et al., 2008).

Virus	Species	Abbr.	Major references
<b>α-Herpesviruses</b>			
<b>- Simplexviruses</b>			
Herpes simplex virus type 1	human	HSV-1	Horsburgh et al., 1999a, b; Nagel et al., 2008; Saeki et al., 1998; Stravropoulos & Strathdee, 1998; Tanaka et al., 2003
Herpes simplex virus type 2	human	HSV-2	Meseda et al., 2004
<b>- Varicelloviruses</b>			
Varicella-zoster virus	human	VZV	Nagaike et al., 2004; Tischer et al., 2007; Wussow et al., 2009; Yoshii et al., 2007; Zhang et al., 2007
Simian varicella virus	rhesus	SVV	Brazeau et al., 2011; Gray et al., 2011
Pseudorabies virus	porcine	PrV	Smith & Enquist, 1999
Bovine herpesvirus type 1	bovine	BHV-1	Mahony et al., 2002; Robinson et al., 2008
Equine herpesvirus type 1	equine	EHV-1	Rudolph & Osterrieder, 2002; Rudolph et al., 2002
Canine herpesvirus	canine	CHV	Strive et al., 2006
Feline herpesvirus type 1	feline	FHV-1	Costes et al., 2006; Tai et al., 2010
<b>- Mardiviruses</b>			
Marek’s disease virus	turkey	MDV	Schumacher et al., 2000
Turkey herpesvirus	turkey	HVT	Baigent et al., 2006
<b>β-Herpesviruses</b>			
<b>- Cytomegaloviruses</b>			
Human cytomegalovirus	human	hCMV	Borst et al., 1999; Dulal et al., 2009; Hahn et al., 2002, 2003; Marchini et al., 2001; Murphy et al., 2003; Sinzger et al., 2008
Rhesus cytomegalovirus	rhesus	rhCMV	Chang & Barry, 2003
Murine cytomegalovirus	murine	mCMV	Messerle et al., 1997
Guinea pig cytomegalovirus	guinea pig	gpCMV	McGregor & Schleiss, 2001a
<b>- Roseoloviruses</b>			
Human herpesvirus 6	human	HHV-6	Borenstein & Frenkel, 2009
<b>γ-Herpesviruses</b>			
<b>- Lymphocryptoviruses</b>			
Epstein-Barr virus	human	EBV	Delecluse et al., 1998
<b>- Rhadinoviruses</b>			
Kaposi-Sarcoma associated herpesvirus	human	KSHV	Delecluse et al., 2001; Zhou et al., 2002
Rhesus rhadinovirus	rhesus	RRV	Estep et al., 2007
Murine γ-herpesvirus 68	murine	MHV-68	Adler et al., 2000; Song et al., 2005
Herpesvirus saimiri	saimiriine	HVS	White et al., 2003; Toptan et al., 2010
<b>Alloherpesviruses</b>			
Koi herpesvirus	carp	KHV	Costes et al., 2008
<b>Poxviruses</b>			
Vaccinia virus		VAC	Cottingham et al., 2008; Domi & Moss, 2002; Meissinger-Henschel et al., 2011
Cowpox virus	bovine	CPXV	Roth et al., 2011
<b>Coronaviruses</b>			
Transmissible gastroenteritis coronavirus	porcine	TGCV	Almazan et al., 2000
Severe acute respiratory syndrome coronavirus	human	SARS CoV	Almazan et al., 2006
Coronavirus OC43	human	OC43	St. Jean et al., 2006
Coronavirus NL63	human	NL63	Donaldson et al., 2008

Table 1. List of cloned viral bacterial artificial chromosomes with key references.

### 3. Manipulation of viral bacterial artificial chromosomes

The generation of a molecular viral BAC clone forms the prerequisite for efficient virus mutagenesis. The site-directed manipulation of single-copy plasmid DNA in *E. coli* by homologous recombination was established in several conditionally or transiently expressed forms. Alternatively, transposon mutagenesis can be used as a non-directed random method.

#### 3.1 Site-directed mutagenesis of viral bacterial artificial chromosomes

Common techniques for the rapid and targeted DNA mutagenesis are based on Red recombination or RecET cloning encoded by prophages  $\lambda$  or  $\phi$ 80, respectively (Court et al., 2002; Lee et al., 2001; Murphy, 1998; Muirers et al., 1999, 2001, 2004; Yu et al., 2000; Zhang et al., 1998). Recombination-mediated genetic engineering (termed "recombineering") via Red and RecET allows almost unlimited modifications of large BAC-cloned DNA sequences in *E. coli* and is widely used in functional genomics (Copeland et al., 2001; Muirers et al., 2001; Narayanan et al. 1999; Sawitzke et al., 2007; Sharan et al., 2009; Thomason et al., 2007; Warming et al., 2005). The Red-recombination system from phage  $\lambda$  consists of the 5'-3'-exonuclease Exo and of the single-strand (ss) DNA-binding protein Beta. These proteins mediate the recombination between dsDNA ends and homologous target sequences on replicating DNA molecules in *E. coli* and are biologically responsible for the integration of  $\lambda$  phage DNA into the bacterial chromosome (Carter & Radding, 1971). Exo acts on dsDNA ends to generate 3'-ssDNA sticky ends (Little, 1967). Then, Beta recognizes the recessed ssDNA ends and anneals them to complementary ssDNA in preformed replication forks leading to their recombination with the homologous sequence (Kmiec & Holloman, 1981; Muniyappa & Radding, 1986). The Red genes are expressed together with the  $\lambda$  *gam* gene under a temperature-inducible promoter for the efficient induction and DNA manipulation in bacteria. Gam is a natural inhibitor of the *E. coli* RecBCD exonuclease, which rapidly degrades dsDNA invading into bacteria (Karu et al., 1975; Murphy, 1991, 1998, 2007; Yu et al., 2000).

While the Red recombination system is expressed, a linear DNA fragment with 40-50 bp homologous flanking regions is inserted into the selected target sequence by Exo and Beta, whereas Gam blocks the RecBCD enzyme from degrading dsDNA ends. The Red system does not need the *E. coli* RecA protein, which is the main endogenous mediator of homologous recombination in *E. coli* (Murphy, 1998; Yu et al., 2000). Therefore, the Red recombination is useful for the easy manipulation of plasmids or bacterial chromosomes in a *recA*-recombination-deficient *E. coli* background (e.g., DY380-derived strains or GS1783) by linear products of the polymerase chain-reaction (PCR) that were generated with primers containing short homologous target sequences at their 5'-ends (Copeland et al., 2001; Oppenheim et al., 2004; Yu et al., 2000). The application of Red-mediated DNA mutagenesis for BAC mutagenesis was greatly simplified by expressing the *red* and *gam* genes from a defective prophage integrated in the *E. coli*-genome, when the culture temperature is increased from 32 to 42°C, without the need for additional expression plasmids. In comparison to the plasmid coexpression strategy, the  $\lambda$  prophage system is up to 100-fold more efficient in Red recombineering and the Red protein expression is more tightly controlled under the temperature-inducible promoter of the  $\lambda$  prophage (Lee et al., 2001; Yu et al., 2000). This more stringent control also reduces the risk for unwanted recombination



during bacterial DNA replication. During the mutagenesis procedure, the mutation of interest is introduced into the target sequence together with an antibiotic resistance gene. The selection marker may be flanked by recombinase recognition sites in order to allow its secondary excision from the recombinants (Lee et al., 2001; Yu et al., 2000). The remaining single copy of a recombinase recognition site limits further repeated steps of the procedure and may also interfere with gene functions in tightly packed genomes. The site-directed mutagenesis may be used to delete further non-essential regions if the cloning capacity is limited in particularly large virus genomes. In addition, genetic elements of the BAC constructs can even be moved within the BAC by site-directed mutagenesis to optimized insertion positions (Wussow et al., 2009).

### 3.2 Two-step *en passant* mutagenesis

By the combination of homologous recombination steps, “traceless”, “seamless”, or “markerless” recombineering strategies were developed which allow the highly efficient mutagenesis of BAC DNA in *E. coli* without retaining any operational sequences (Sawitzke et al., 2007; Sharan et al., 2009; Thomason et al., 2007; Tischer et al., 2006, 2010a, b; Warming et al., 2005). The *en passant* procedure combines Red recombination with cleavage by the homing endonuclease I-SceI (Tischer et al., 2006, 2010a, b). The asymmetrical 18 bp I-SceI recognition site can be inserted into plasmid DNA and cleaved after induced expression of the homing enzyme. This highly sequence-specific restriction endonuclease produces dsDNA ends accessible for homologous recombination (Jamsai et al., 2003). For the *en passant* protocol, large oligonucleotide primers are designed which allow generation of a PCR product for the Red-mediated insertion of a selection marker together with an I-SceI recognition site flanked by a 50 bp direct sequence duplication. After selection of recombinants, a double-strand break is induced by I-SceI cleavage at the respective recognition site. This permits the seamless excision of the positive selection marker (psm) by a second Red-mediated homologous recombination event via short duplicated sequences. *En passant* mutagenesis can be used to generate single point-mutations, substitutions, deletions, or insertions, e.g., of expression constructs, epitope tags, or autofluorescent fusion proteins (Figure 2). For the generation of single point mutations, a fragment including a psm and an I-SceI site is amplified with primers adding 60-80 bp extensions to the psm-I-SceI unit. The distal 40-50 bp of the primer sequences and of the resulting PCR fragments are homologous to the target site in the BAC. Additionally, 40-50 bp of the mutated target site are included into both primers in reverse complementary orientation. The psm fragment with the appropriate flanking sequence duplication and point mutation is inserted into the site of interest by the first Red recombination step. After induction of I-SceI cleavage, the psm is excised between the duplicated sequences and the markerless point mutation is generated by the second Red recombination step (Figure 2A). Similar procedures are used for large deletions. In this case, the PCR-primers for psm-I-SceI amplification carry 5'-extensions from the up- and downstream regions flanking the deletion area (Figure 2C). For the seamless insertion of large sequences by *en passant* mutagenesis, a cassette containing the psm and the I-SceI site and a 40-50 bp sequence duplication is PCR amplified. This PCR fragment is then inserted into a unique restriction endonuclease site of the cloned sequence of interest. The fragment is released from the plasmid by terminal restriction endonuclease sites and used for the precise insertion into the target region by Red recombination. In selected recombinants, the psm is seamlessly removed by the second *en passant* recombination of the 50 bp duplication of the inserted sequence of interest (Figure 2B).

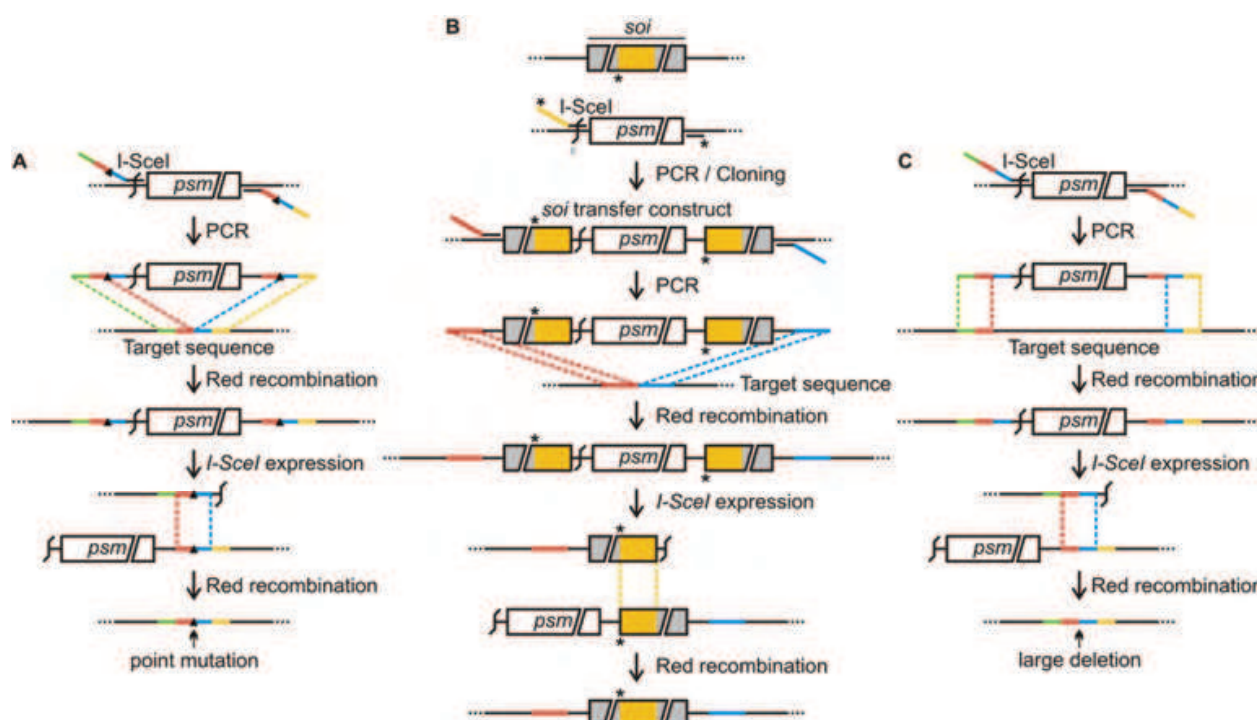


Fig. 2. *En passant* mutagenesis. A) Point mutation. A positive selection marker (psm) and an I-SceI site are PCR-amplified with primers carrying 60-80 bp homologous extensions (coloured elements). In both primers, 40-50 bp (red and blue) around the core sequence are reverse complementary and carry the mutation (triangles). The PCR product is inserted into the target site by Red recombination. After a double-strand break by I-SceI, the psm is excised by Red recombination of the duplication, resulting in the precise point mutation. B) Insertion of large sequences. The psm-I-SceI cassette is amplified using one primer with a 40-50 bp duplication (yellow) and inserted into a unique restriction site (\*) of a cloned sequence of interest (soi). The soi transfer construct is then amplified using primers with 40-50 bp extensions (red and blue) and inserted into the target site by Red recombination. After I-SceI and red expression, the psm is deleted from soi by recombination of the short duplications. C) Large deletion. The psm-I-SceI element is amplified using primers with 5'-ends homologous to adjacent sequences from the deletion region (coloured). The soi is then deleted by Red recombination. The procedure follows the further steps as in panel A (adapted from Tischer et al., 2006; Felix Wussow, Ph.D. thesis, Christian Albrecht University of Kiel, 2009).

The well-established and highly versatile markerless manipulation techniques allow for the repetitive manipulation of the cloned genomes even within the direct or inverted viral repeat sequences. This unique feature makes the BAC technology especially useful to mutagenize elements involved in DNA replication or maturation, *e.g.*, the origin of DNA replication or DNA packaging signals, which are usually present in the repeat sequences or in the genomic termini of herpesviral genomes. Similarly, many DNA elements relevant for the establishment of latency and the reactivation from the latent state as well as for the integration of the viral DNA into the host genome are located in the viral repeats and can be efficiently studied by the BAC technology.

The *en passant* mutagenesis strategy can also be used for the seamless removal of the BAC-vector sequences from the viral genomes during virus reconstitution in eukaryotic cells

(Figure 3). Appropriately designed viruses will delete the mini-F element due to homologous recombination of duplications of viral genome fragments or due to intrinsic genome features. Such strategies were based on genomic duplications engineered in direct orientation at either site of the vector elements (Strive et al., 2007; Wagner et al., 1999). However, such BACs with direct duplications can apparently not be stably maintained in *E. coli* with the temperature-inducible Red expression cassette on a defective  $\lambda$  prophage integrate, since the mini-F vector will be lost presumably by homologous recombination between the duplicated viral sequences, even in the non-induced state. This has been overcome by providing the ET cloning functions from an additionally transfected plasmid for the efficient removal function after the mutagenesis procedure (Strive et al., 2007; Wagner et al., 1999), although recombineering mediated by plasmid-encoded functions is up to 100-fold less efficient than the integrated  $\lambda$  prophage system (Lee et al., 2001; Muylers et al., 1999; Narayanan et al., 1999; Yu et al., 2000). In another  $\lambda$ -based self-excision system (Figure 3), the duplicated viral sequences flanking the mini-F integration site were inserted in inverse orientation. This arrangement allowed the stable maintenance of the BAC DNA in

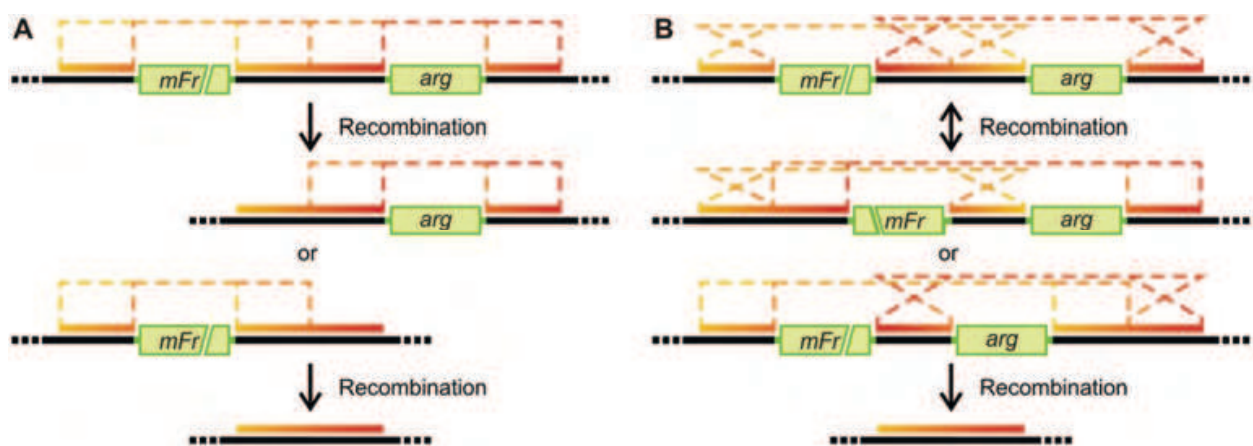


Fig. 3. Self-excision of BAC sequences by stabilized genomic duplication. A) BAC vector (green) self-excision from the viral DNA (black) by a forward genomic duplication (yellow to red gradient bars) inserted between the mini-F replicon (mFr) and the antibiotic resistance gene (arg). Two recombination events are necessary to release the mFr or the arg from the viral DNA. B) Self-excision of the BAC sequences from the viral DNA by a genomic duplication inserted in inverse orientation into the vector elements. Two successive events of recombination are required for the complete BAC vector excision from the viral DNA (adapted from Felix Wussow, Ph.D. thesis, Christian Albrecht University of Kiel, 2009).

*E. coli*, since two successive events of homologous recombination would be required for the deletion of the complete BAC sequences. BACs with these inverse viral duplications flanking the mini-F integration site within a viral direct S-repeat area did not lead to any detectable BAC instability in the recombineering *E. coli* strain GS1783 and allowed the efficient deletion of the vector moiety (Tischer et al., 2007).

Mini-F vector sequences containing inverse genomic duplications that were inserted into different essential viral replication genes also allow the efficient mini-F vector self-excision. Alternatively, certain repeat regions or terminal virus sequences are suitable under specific conditions for the autonomous vector excision (Tischer et al., 2007; Wussow et al., 2009; Zhou et al., 2010). Different genomic insertion sites for the mini-F vector were compared for the seamless reconstitution of recombinant virus. The most efficient variant pHJOFpac



carried the mini-F vector insertion at the terminal genomic junction of VZV, which is an optimal vector integration site permitting the rapid and spontaneous generation of recombinant progeny devoid of any vector elements (Wussow et al., 2009). A similar integration site was described for rhesus rhadinovirus (RVV), from which the vector is also efficiently released (Zhou et al., 2010). Therefore, the terminal genomic junction might be in general an optimal integration site for the mini-F vector to construct other large linear viral DNA genomes as infectious BACs. In addition, the recombineering methodology even allows the transposition of genetic elements to defined new locations within the same BAC molecule (Wussow et al., 2009). Thus, the mini-F-transposition strategy eliminated the last hurdle to perform any imaginable kind of targeted seamless BAC modifications in *E. coli*. This is in general a valuable tool to reorganize or repair any other established BACs, e.g., for the development of gene therapy or vaccine vectors or of specific targeting vectors for conditional knock-out mice.

### 3.3 Transposon mutagenesis of viral BACs

Alternatively, the random and non-directed approach of transposon mutagenesis was adapted for virus BAC mutagenesis (Brune et al., 1999; Smith & Enquist, 1999) and provides saturated libraries of diverse recombinant mutants. The random transposon mutagenesis was successfully performed for the large genomes of hCMV, mCMV, equine herpesvirus type 1 (EHV-1), and murine herpesvirus type 68 (MHV-68) (Bubeck et al., 2003; Hansen et al., 2006; Hobom et al., 2000; Song et al., 2005; Yu et al., 2003). Fortunately, the transposon insertion occurs preferentially into plasmid DNA in comparison to the bacterial genome. After transposon mutants have been tested for their functional phenotype, the respective genotype must be determined. This is accomplished using PCR primers which bind to the transposon insert and allow an easy genome-wide mapping and specific sequencing from the viral BAC genome. The major advantages of transposon mutagenesis are the unbiased random approach and the rapid generation of large BAC mutant libraries. However, this may be complicated by multiple insertions in the same BAC or by an uneven distribution of the insertion sites over the virus genome.

### 3.4 Functional mutagenesis of specific viral BACs

BAC generation and mutagenesis has been reported on numerous viruses (Table 1). HSV-1, the prototype genome of the herpesviruses in general, as well as of the  $\alpha$ -herpesviruses and simplexviruses in particular, exists as BACs from different virus strains (Horsburgh et al., 1999a, b; Nagel et al., 2008; Saeki et al., 1998; Stavropoulos & Strathdee, 1998; Tanaka et al., 2003). Many BAC-based studies were performed on mutations in HSV-1 genes (e.g., Boutell et al., 2002; Legee et al., 2009; O'Hara et al., 2010; Roberts et al., 2009; Tong & Stow, 2010). Also, HSV-2 is available as a viral BAC (Meseda et al., 2004). Several strains of the highly cell-associated varicellovirus prototype, VZV, were cloned as infectious BACs, the vaccine and parental OKA strains, as well as the wild-type isolate HJO (Nagaike et al., 2004; Tischer et al., 2007; Wussow et al., 2009; Yoshii et al., 2008; Zhang et al., 2007, 2008). A VZV BAC with a luciferase reporter gene allowed viral replication studies *in vivo* (Zhang et al., 2007, 2008). VZV was also subjected to saturating mutagenesis for determining essential genes for viral replication (Zhang et al., 2010). The genome of the closely related simian varicella virus (SVV) of rhesus monkeys has also been made available as a BAC (Brazeau et al., 2011; Gray et al., 2011). Pseudorabies virus (PrV) is another varicellovirus with highly important model



function for herpesvirus biology. PrV was cloned as a BAC and used for pathogenesis studies *in vivo* (Smith & Enquist, 1999, 2000; Fuchs et al., 2009; Kopp et al., 2004]. Additional varicellovirus BACs of various animals have been studied *in vitro* and *in vivo*: Bovine herpesvirus type 1 (BHV-1; Gabev et al., 2009; Mahony et al., 2002; Robinson et al., 2008; Trapp et al., 2003), EHV-1 (Goodman et al., 2007; Rudolph & Osterrieder, 2002; Rudolph et al., 2002; Yao et al., 2003), canine herpesvirus (CHV; Strive et al., 2006), and feline herpesvirus type 1 (FHV-1; Costes et al., 2006; Richter et al., 2009; Tai et al., 2010). Moreover, BACs exist for the mardiviruses Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) (Baigent et al., 2006; Petherbridge et al., 2003; Schumacher et al., 2000; Zhao et al., 2008). The particularly large BAC-cloned koi herpesvirus belongs to the alloherpesviruses and not to the typical  $\alpha$ - to  $\gamma$ -herpesviruses (Costes et al., 2008, 2009).

The prototype for the  $\beta$ -herpesviruses, hCMV, has the largest genome among the human herpesviruses containing approximately 165 genes. Clinical hCMV isolates have larger genomes and replicate well in macrophages and endothelia cells, whereas the laboratory strains have undergone deletions and replicate efficiently only in fibroblasts (Dolan et al., 2004). Therefore, after the laboratory strain AD169, various laboratory strains and clinical isolates were cloned as infectious BACs in order to provide defined genetic conditions for functional studies (Borst et al., 1999; Dulal et al., 2009; Hahn et al., 2002, 2003; Marchini et al., 2001; Murphy et al., 2003; Sinzger et al., 2008). Many functional studies were performed with hCMV BACs (*e.g.*, Britt et al., 2004; Spaderna et al., 2005). Saturating random mutagenesis over the entire hCMV genome was performed by transposon insertion (Hobom et al., 2000; Yu et al., 2003). Moreover, the necessity for virus replication was determined for 162 individual hCMV genes (Dunn et al., 2003). mCMV as an important animal model for hCMV pathogenesis was the first herpesvirus genome to be cloned as an infectious BAC (Messerle et al., 1997) which has been used for *in vitro* and *in vivo* mCMV studies (*e.g.*, Wagner et al., 1999; Cicin-Sain et al., 2003, 2007; Menard et al., 2003; Schnee et al., 2006). BAC-clones have also been constructed for the genomes of rhesus CMV (rhCMV; Chang & Barry, 2003; Lilja et al., 2008; Rue et al., 2004) and guinea-pig CMV (gpCMV; Crumpler et al., 2009; McGregor & Schleiss, 2001a; Schleiss, 2008). The BAC of the human roseolovirus HHV-6 is still dependent on a helper virus infection (Borenstein & Frenkel, 2009; Borenstein et al., 2010).

The oncogenic  $\gamma$ -herpesvirus and lymphocryptovirus EBV (Delecluse et al., 1998) was one of the first cloned viral BACs and many studies have applied this technique, *e.g.*, on oncogene functions (Ahsan et al., 2005; Anderton et al., 2008; Chen et al., 2005; Kanda et al., 2004). The oncogenic rhadinovirus KSHV is hampered by its non-efficient replication in cell culture. Also for KSHV, several BACs were constructed and applied for functional analyses (Delecluse et al., 2001; Fan et al., 2006; Lu et al., 2010; Lukac et al., 2001; Luna et al., 2004; Majerciak et al., 2007; Xu et al., 2005, 2006; Yakushko et al., 2011; Zhou et al., 2002). Additionally, the major rhadinovirus animal model viruses were cloned in BACs, such as RRV (Estep et al., 2007; Zhou et al., 2010), MHV-68 (Adler et al., 2000; Pavlova et al., 2003), and herpesvirus saimiri (HVS; Calderwood et al., 2005; Toptan et al., 2010; White et al., 2003, 2007).

Besides the herpesviruses, BAC-cloning has been successfully applied for the poxvirus Vaccinia virus (Cottingham et al., 2008; Domi & Moss, 2002; Meissinger-Henschel et al., 2011) and cowpox virus (Roth et al., 2011). Moreover, this method was useful to generate full-length molecular clones of the large RNA genomes of different coronaviruses such as the

porcine virus of transmissible gastroenteritis, the severe acute respiratory syndrome coronavirus and the human coronaviruses NL63 and OC43 (Almazan et al., 2000, 2006; Donaldson et al., 2008; St. Jean et al., 2006).

#### 4. Conclusion

The viral DNA replication strategies were very well exploited for the generation of efficient BAC cloning, mutagenesis and reconstitution techniques. BAC cloning and recombineering strategies are essential for the efficient mutagenesis and analysis of herpesviral and poxviral gene functions and reduce problems due to unwanted mutations outside of the region of interest. In addition, only the existence of a cloned full-length genome guarantees the usage of defined genome structures, especially in variable virus genes. For example, the BAC-mediated expression of fusion proteins of viral factors with autofluorescent moieties can be used for analysing the expression and localization patterns of viral functions (*e.g.*, Antinone & Smith, 2006). The precise, seamless, and repetitive manipulation of repeat regions may facilitate the study of viral DNA elements involved in DNA replication, genome maturation, and packaging, as well as in latency, reactivation, and chromosomal integration.

Besides basic research, the BAC technology has its major translational applications in vector and vaccine development. In the case of viral vector design, the recombineering technique allows the easy deletion of useless non-essential regions or virulence genes from the viral vector genome (*e.g.*, Cicin-Sain et al., 2007). This may reduce unwanted side-effects and may increase the cloning potential for transgenes. Even large and complex multi-unit transgene cassettes can be inserted as well as novel conditional replication and expression control systems (*e.g.*, Glass et al., 2009). Such viral vectors include optimized transgene cassettes, the lack of genomic integration, and the advantage of physiologic infection routes. This may also comprise cell-type specific functions as for example in EBV or HVS vectors for B- or T-lymphocytes, respectively. Viral vectors may be constructed as efficiently replicating oncolytic agents (Kuroda et al., 2006; Marconi et al., 2009; Terada et al., 2006) or as packaging-cell dependent transduction vehicles (*e.g.*, Hettich et al., 2006).

The BAC technology has also provided new possibilities for vaccine development. For example, EHV-1 BAC-derived viruses were constructed for immunization against West Nile virus, bovine diarrhea virus or Venezuelan equine encephalitis virus (Rosas et al., 2007a, b, 2008). Especially for possible vaccination strategies for hCMV, the detailed analysis of the animal model viruses is important. In the case of mCMV and gpCMV, such BAC-based immunization strategies have shown promising results (Cicin-Sain et al., 2003, 2007; Crumpler et al., 2009; Redwood et al., 2005). In the rhesus monkey model, the viral inhibition mechanism for secondary rhCMV infections suggests respective strategies for hCMV vaccines (Hansen et al., 2010). Such observations may lead the way to novel recombinant human vaccines, for example by replication-deficient hCMV or HSV-1 (Schleiss et al., 2006; Suter et al., 1999).

Although the highly efficient modified vaccinia virus Ankara (MVA) has already been well established as a vaccine for the application in humans, the BAC strategy shows considerable advantage for optimizing novel vaccine generations (Cottingham et al., 2008; Domi & Moss, 2002; Meissinger-Henschel et al., 2011; Roth et al., 2011), especially since MVA and related

poxviruses are also useful as transgene vectors for the efficient immunization against heterologous pathogens.

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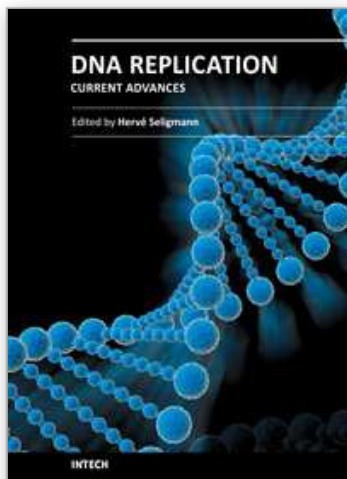


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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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