# Mutagenesis of the Cytomegalovirus Genome

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**Abstract** Bacterial artificial chromosomes (BACs) are DNA molecules assembled in vitro from defined constituents and are stably maintained as one large DNA fragment in *Escherichia coli*. Artificial chromosomes are useful for genome sequencing programs, for transduction of DNA segments into eukaryotic cells, and for functional characterization of genomic regions and entire viral genomes such as cytomegalovirus (CMV) genomes. CMV genomes in BACs are ready for the advanced tools of *E. coli* genetics. Homologous and site-specific recombination, or transposon-based approaches allow for the engineering of virtually any kind of genetic change.

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## Introduction

Animal viruses use a small set of genes to profoundly affect functions of complex hosts. The goal of virus genetics is to understand virus-host interactions at the molecular level. Collection or construction of virus mutants is obligatory for this goal. Until recently, the manipulation of herpesvirus genomes was bound to construction of mutants by homologous recombination in infected cells. Bacterial artificial chromosomes (BACs) are large, circular single-copy episomes of *Escherichia coli* which are suited for maintenance of very large foreign DNA fragments including genomes of large DNA viruses, as first demonstrated by Luckow and colleagues for baculovirus (Luckow et al. 1993). In 1997, we pioneered the BAC-based genetic analysis of herpesviruses with the first example of the cloning and mutagenesis of infectious mouse CMV (MCMV) genome in *E. coli* (Messerle et al. 1997). BAC technology has become a general approach in herpesvirus genetics and beyond for analysis of large and/or unstable viral genomes (Almazan et al. 2000; Ruzsics et al. 2006). This review discusses the different strategies for generation of recombinant herpesviruses and shows the potential of BAC-based herpesvirus genetics. Examples are taken mainly from human CMV (HCMV) and MCMV; reference is also given to work on other herpesviruses.

## CMV Genetics in Cells

### *Forward (Classical) Genetics*

The objects of viral genetics are the mutant alleles, where changes in genetic material result in phenotypic alterations that can be analyzed. The frequency of spontaneous mutations in the DNA genomes of herpesviruses ranges between $10^{-8}$ and $10^{-11}$ per incorporated nucleotide. Therefore, mutagens have been used to increase the rate of mutations during virus replication by a procedure called in vivo mutagenesis (Schaffer 1975; Schaffer et al. 1984). The characterization of conditional alleles, such as temperature sensitive (*ts*) mutations, has been favored, because both isolation of mutants and their analysis required operational viability. *Ts* mutants are a result of a missense point mutation that alters the primary amino acid sequence of the encoded protein, leading to a loss of function only at a higher (restrictive) temperature. The demanding step is the genetic mapping of the causative mutations. The methods for genetic mapping of *ts* mutations, such as cross-complementation and marker rescue assays, are time-consuming and work-intense. Yet, until recently, *ts* mutants were the only generally applicable tools for studying null phenotypes of essential herpesvirus genes.

Chemical mutagenesis has also been applied to HCMV and MCMV genetics. Early work on HCMV led to the classification of complementation groups

(Yamanishi and Rapp 1977; Ihara et al. 1978). Only few HCMV *ts* mutants could be mapped and associated with open reading frames (ORFs) (Dion et al. 1990; Ihara et al. 1994). The analysis of MCMV *ts* mutants has mainly centered on direct in vivo studies using mutants without mapping (Akel et al. 1993; Akel and Sweet 1993; Bevan et al. 1996; Gill et al. 2000). To our knowledge, none of the *ts* mutations of MCMV has so far been associated with a specific genetic locus. Recently, targeted *ts* mutants for the HCMV IE 2 gene were constructed using BAC technology (Heider et al. 2002).

## *Reverse Genetics*

The ability to clone viral DNA fragments, to propagate and manipulate those recombinant plasmids in *E. coli*, opened several opportunities for the analysis of isolated viral genes. Subcloned viral genes, after mutation and functional analysis in vitro, could be reintroduced into the viral genome to investigate the phenotype in the genomic context (reverse genetics). First, the herpes simplex virus (HSV) genome was used to construct site-directed genome mutants (Mocarski et al. 1980; Smiley 1980; Post and Roizman 1981). After the availability of sequences, site-directed mutagenesis also became an option for CMVs (Spaete and Mocarski 1987; Manning and Mocarski 1988). In principle, a marker gene is introduced into the viral genome by homologous recombination in infected cells thereby disrupting or deleting a viral gene (Fig. 1a). The recombination is under the control of the cellular recombination machinery. It is a rare event and the *wt* virus dominates the resulting progeny pool. Therefore, labeling or even positive selection of the mutants is essential for their isolation by markers such as the β-galactosidase (Spaete and Mocarski 1987; Manning and Mocarski 1988), the neomycin resistance gene (Wolff et al. 1993), and the xanthine guanine phosphoribosyltransferase (gpt) gene (Mulligan and Berg 1981; Greaves et al. 1995).

The advent of cosmid vectors with a capacity to maintain larger DNA fragments (20-50 kbp) in *E. coli* led to cloning of the HSV genome as a set of overlapping cosmid clones (van Zijl et al. 1988). Infectious *wt* virus is reconstituted after co-transfection of the overlapping cosmid set into permissive cells via multiple homologous recombination (Fig. 1b). For mutagenesis, the genetic change is introduced into one of the cosmid fragments. The mutant virus is reconstituted by co-transfection of the mutated cosmid clone with the other cosmids. HSV cloning was followed by cosmid-based reconstruction of HCMV and MCMV (Kemble et al. 1996; Ehsani et al. 2000). The utility of cosmids for generation of mutant CMVs was demonstrated by generation of 17 different UL54 mutants of HCMV (Cihlar et al. 1998). The huge advantage of the cosmid approach over the direct recombination methods is the absence of the *wt* genome. The size of the cloned viral fragment to be mutated allows standard in vitro mutagenesis techniques applicable for plasmids. However, the genetic instability of the system during

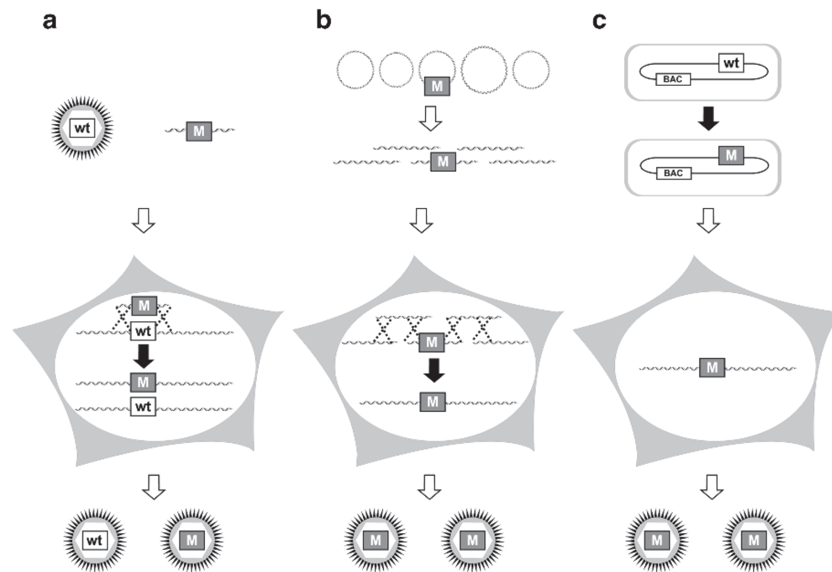


**Fig. 1** Different methods of herpesvirus mutagenesis. **a** Site-directed mutagenesis in eukaryotic cells. A linear DNA fragment containing a marker along with the mutation (*M*) flanked by homologies to the viral target sequence is transfected into virus infected cells. By homologous recombination (*dashed lines*) the marker gene and the mutation insert into some of the virus genomes deleting the wild type sequence (wt) Recombinant viruses and wild type viruses need further separation. **b** Cosmid mutagenesis in eukaryotic cells. Overlapping viral fragments spanning the entire genome are cloned as cosmids. A mutation (*M*) is introduced into one fragment. After transfection of the linearized cosmid clones into permissive cells, the virus genome can be reassembled by several homologous recombination steps generating the mutant virus. **c** Principle of the mutagenesis with bacterial artificial chromosomes (BACs) in *E. coli*. Recombinant viral BACs can be generated using various site-directed and random mutagenesis approaches. Recombinant viral BAC DNA with a mutation (*M*) is then transfected into permissive eukaryotic cells and the mutant virus progeny is thereby reconstituted

virus reconstitution became an issue (Horsburgh et al. 1999). Since virus reconstitution relies on several recombination events in eukaryotic cells, changes in the recombinant genomes may occur (Kemble et al. 1996). The cosmid-based mutagenesis is based on recombination in cells and requires the regeneration of a replication-competent virus genome. Therefore, mutant genomes in which an essential gene is affected are difficult, if not impossible, to construct. Unfortunately, revertants cannot be constructed without unreasonable efforts, since the generation of a revertant in its strict sense would require cosmid cloning of the newly generated recombinant genome. Therefore, the usage of this elegant method was restricted to mutagenesis of genes with a known phenotype.

## CMV Genetics in Bacteria

### *Bacterial Artificial Chromosomes*

The desire to clone large eukaryotic genomes in order to acquire contiguous physical chromosome maps brought emphasis to cloning vectors of larger insert capacity. Although yeast artificial chromosomes (YACs) can encompass DNA fragments larger than 1000 kbp, YACs are marred by spontaneous rearrangements, insert instability and yeast DNA contamination (Ramsay 1994; Schalkwyk et al. 1995). Stable maintenance of foreign DNA larger than 300 kbp in size was reported using either a fertility factor (F-factor) replicon based bacterial artificial chromosome (BAC) (Shizuya et al. 1992) or a bacteriophage P1 replicon-based cloning system called PAC (Ioannou et al. 1994). In contrast to YACs or cosmids, the BAC clones show surprising sequence stability in appropriate strains of *E. coli*. As a rule, most of the useful BAC hosts are derivatives of DH10B, pointing to the importance of the genetic background of the *E. coli* host strain (Shizuya et al. 1992; Tao and Zhang 1998). Human genome fragments as BACs were maintained over 100 generations in bacteria without detectable changes (Shizuya et al. 1992). The strict control of the F-factor-based replicon keeps one copy of the BAC per cell and reduces recombination events via repetitive DNA elements present in the eukaryotic DNAs. BACs now play a central role in genome research.

### *Cloning and Maintenance of CMV Genomes as BACs*

We pioneered BAC cloning and mutagenesis of MCMV (Messerle et al. 1997). This concept was quickly taken up by several groups. By now, many genomes of many herpesviruses, including different strains of CMVs from various species, have been cloned (for review see Brune et al. 2000). The construction of a herpesvirus BAC starts with conventional mutagenesis procedures. First, the BAC vector sequences flanked with appropriate viral sequences are introduced into the genome by homologous recombination in cells. The linear double-stranded DNA genome of herpesviruses circularizes after infection and these replication intermediates of the BAC-containing herpesvirus genome are transferred by transformation into *E. coli*. This transformation step is needed only once. In *E. coli*, virus functions do not need to be expressed for either genome amplification or mutagenesis procedures. Potential size constraints of the CMV genomes with regard to packaging limits due to the oversize of the inserted BAC cassette can be solved by deletion of nonessential genomic sequences. (Messerle et al. 1997; Borst et al. 1999; Wagner et al. 1999). The deleted sequences can be reinserted after the cloning procedure (Wagner et al. 1999). To regenerate infectious virus, the herpesvirus BACs are transfected into permissive host cells (Fig. 1c). Herpesvirus genomes in BACs are ready for the advanced tools of *E. coli* genetics, which include homologous and site-specific

recombination, or transposon-based methods. Here, the techniques have often been pioneered by research labs outside the field of virology and are discussed as they have been adapted to CMV genetics.

## *Allelic Exchange by Shuttle Plasmid Mutagenesis*

Allelic exchange by shuttle plasmids shares aspects of conventional reverse herpes-virus genetics. The desired mutation is cloned into a temperature-sensitive suicide plasmid and flanked by viral sequences homologous to the genomic target site. The shuttle plasmid is then transformed into the BAC carrying *E. coli*, which expresses RecA. The exchange between the mutated and *wt* sequence requires two homologous recombination events (Fig. 2). In the first step, the shuttle plasmid and the BAC recombine via one homology arm resulting in a co-integrate. At the nonpermissive

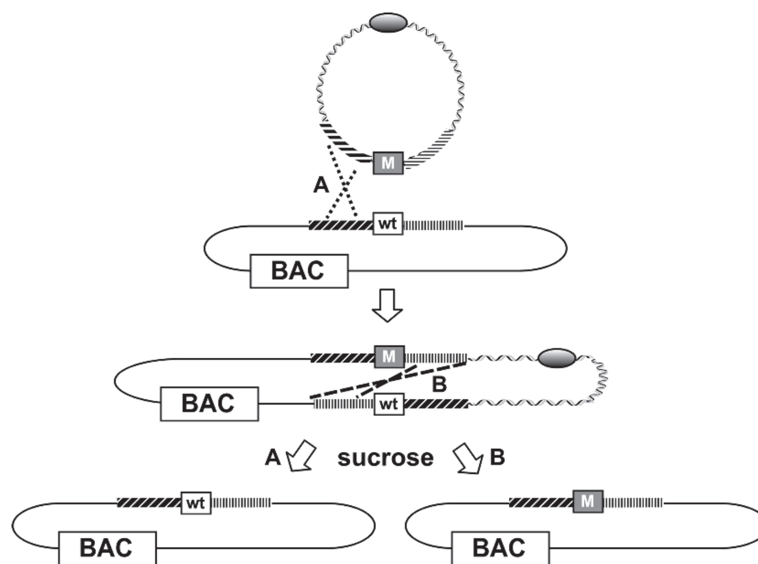


**Fig. 2** Allelic exchange using shuttle plasmids. A temperature-sensitive suicide shuttle plasmid that contains the desired mutation (*M*) and viral homologies up- and downstream to the viral target sequence (*dashed lines*) is transformed into *E. coli* carrying the viral BAC. RecA-mediated homologous recombination via one homology arm (*A*) leads to cointegrate formation, which is selected by the antibiotic-resistance genes of the viral BAC and the shuttle plasmid. Free shuttle plasmids are lost at a nonpermissive temperature. In the second recombination step, the cointegrate is resolved. Recombination via the same homology arm (A) leads to a wild type (wt) viral BAC (*on the left*), recombination via the other homology arm (*B*) leads to generation of mutant (M) viral BAC (*on the right*) Unresolved cointegrates and shuttle plasmids are eliminated by sucrose counter selection against SacB (*shaded sphere*), which is present in the shuttle plasmid backbone

temperature the nonintegrated shuttle plasmid is lost. Then the selected co-integrate is resolved by a second recombination event (Fig. 2). There are two possibilities: if the second recombination occurs via the homology arm of first step, *wt* BAC is reconstituted. If the second recombination occurs via the other homology arm, a mutant is gained. Only a minority of the co-integrates undergo RecA-mediated resolution (O'Connor et al. 1989; Messerle et al. 1997). The inclusion of sacB (Steinmetz et al. 1983; Blomfield et al. 1991) in the shuttle plasmid allows counter-selection against unresolved co-integrates. Several herpesvirus mutants have been generated using this method (Angulo et al. 1998, 2000a, 2000b; Wagner et al. 1999, 2000; Hobom et al. 2000; Brune et al. 2001a; Sanchez et al. 2002). The homologous recombination mediated by RecA prefers long (1-3 kbp) homologies, which need to be cloned along with the mutation into the shuttle plasmid. Therefore, the construction of the shuttle plasmid may need several cloning steps. Shuttle plasmid-based allelic exchange allows the neat introduction of any kind of mutation (point mutation, deletion, insertion, sequence replacement) into a viral BAC without leaving any operational trace in the genome and represents a method of choice when a complex work is concentrated on one specific genomic region.

## *Allelic Exchange Using Linear Fragment Mutagenesis*

Stewart and colleagues described a one-step mutagenesis method called ET recombination, which uses the recombination functions recET from prophage Rac or the functions redαβ from bacteriophage λ for introduction of mutations into a circular DNA by in vitro-generated linear fragments (Zhang et al. 1998; Muyrers et al. 2000). We and others adapted this method to mutagenesis of viral BACs (Adler et al. 2000; Borst et al. 2001; Kavanagh et al. 2001; Schumacher et al. 2001; Dorange et al. 2002; Rudolph and Osterrieder 2002; Strive et al. 2002; Tischer et al. 2002; Wagner et al. 2002). A linear DNA fragment containing a selectable marker and homologous sequences flanking the target site are transferred into recombination proficient *E. coli* carrying the target BAC. It is important to prevent the degradation of the transformed linear DNA. Therefore, either exonuclease-negative bacteria are used or the exonuclease inhibitor red γ from bacteriophage λ is co-expressed with the recombinases. The selectable marker along with the mutation is introduced into the BAC by a double crossover event (Fig. 3). Compared to the RecA-mediated two-step recombination with shuttle plasmids, ET recombination has advantages. The RecET or redαβ expression allows exact recombination between homologies as short as 25-50 nts. Therefore, the homology arms including the mutated sequence can be provided by synthetic oligonucleotide primers, which are used to amplify the selection cassette. This form of BAC engineering is termed ET cloning, ET recombination, recombinogenic engineering, or recombineering. Many systems have been published that use different or altered recombinases and/or different expression systems controlling their expression. Recombineering facilitates many kinds of genomic experiments that have otherwise been difficult

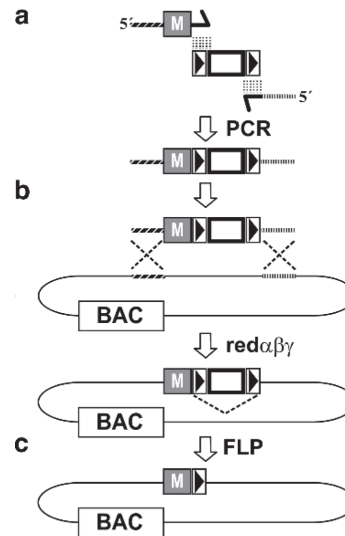


**Fig. 3** Allelic exchange using linear PCR fragments. **a** A selectable marker gene (*open box*) that can be flanked by FRT sites (*black triangle in a square*) is amplified by PCR using a contiguous primer pair (*arrows*) The primers contain homologies of 35-50 nt to the viral target sequence at their 5′-ends (*hatched lines*), a mutation (*M*) and priming regions to the selectable marker gene (*black lines*) **b** The generated linear PCR fragment is transformed into *E. coli* carrying the viral BAC and containing recombinases and an exonuclease inhibitor (redαβγ). The desired mutation along with the selectable marker and the FRT sites is introduced into the viral BAC by double crossover. **c** Additional expression of the site-specific recombinase FLP leads to the excision of the selectable marker reducing the operational sequences to only one FRT site

to carry out. The mutagenesis is independent of specific sequence elements; thus the site of mutagenesis can be freely chosen. A risk is the instability of the viral BAC during this mutagenesis procedure since presence of even short repeated sequences in the target genome can lead to unwanted recombination events. Replacing *wt* sequences with a positive selection marker requires only one recombination step and it is easy to create knockout mutants. In addition, it operates practically without background because the positive selection allows the survival of only the desired recombinants and directly sorts out the genome rearrangements induced by the repeat regions. Recently, a comprehensive set of individual deletion mutants of all HCMV genes have been generated by recombineering for functional profiling of the entire genome (Yu et al. 2003). However, this procedure leaves operational trace in the mutated genomes, namely the bacterial selection marker, which is associated with the risk of unpredictable polar effect on usually complex viral transcription units. To lower the size and the risk of the mutational traces, the selectable marker can be flanked with FRT (FLP recognition target) sites (Cherepanov and Wackernagel 1995). These sites allow excision of the marker in a second step by Flp recombinase in *E. coli* (Fig. 3c), leaving only approximately 70-100 nt extra sequence around the introduced mutation (Wagner and Koszinowski 2004).

From the very beginning of BAC recombineering, attempts were made to construct mutants without operational trace. All strategies that are useful for the size of herpesvirus BACs apply two consecutive steps of homologous recombination. First, a combined marker, which allows both positive and counter-selection in *E. coli*, is introduced at the targeted site, resulting in an intermediate that is isolated by positive selection. Next, by a second round of recombination, the markers are replaced with the desired sequence and the right recombinant can be

enriched by a pressure against the counter-selection marker. This step, however, is error-prone. First of all, there is no counter-selection system known that works with 100% efficiency. This constitutive leakiness of any counter-selection provides a background that can be critical because the efficiency of the ET/red recombination is low. Second, any counter-selection marker can mutate and the mutated alleles will also appear as a background in large-scale cultures of bacteria proficient for recombination. Third, the selection is applied for the loss of the marker; therefore any unwanted rearrangement induced by repeated sequences in the target genome leading to the loss of the marker will produce selectable recombinants. The first two problems are associated with the counter-selection system used. There are two procedures that seem to be efficient enough for recombineering herpesvirus BACs, namely galK and the I-SceI meganuclease-based counter-selection (Warming et al. 2005; Tischer et al. 2006). The risk of genome rearrangements is controlled at best by the fine-tuning of recombinase expression. However, the instability of the BACs is also influenced by the repeat regions within the specific target genomes (Adler et al. 2000; Warming et al. 2005). Unfortunately, herpesvirus BACs abound in repeats of any kind. Not surprisingly, the approaches of traceless recombineering have been tailored to these genomes only long after the first reports on linear fragment mutagenesis of MCMV BAC (Tischer et al. 2006).

## *Transposon Mutagenesis for Reverse and Forward Genetics*

Transposons (Tns) are mobile genetic elements that insert themselves into a DNA molecule (Craig 1997). After transfer of a Tn-donor plasmid into *E. coli*, the Tn can jump into the viral BAC (Fig. 4). The temperature-dependent suicide Tn donor plasmid is eliminated at the restrictive temperature. Some Tns preferentially insert into the negatively coiled plasmids (e.g., Tn1721) and allow direct isolation of mutated BACs (Brune et al. 1999). Others, like Tn5 or Tn10, are less selective and mutated BACs need to be enriched by a retransformation round (Smith and Enquist 1999). The Tn insertion is determined by sequencing from primer sites within the Tn (Brune et al. 1999) (Fig. 4b). Large libraries of mutant BAC genomes can be established and screened for mutants of specific genes or gene families (Fig. 4c). Even a comprehensive library of transposon mutants classified all known genes of HCMV and guinea pig CMV with regard to their influence on the virus growth in vitro (Yu et al. 2003; McGregor et al. 2004).

A support for genetics applications based on large libraries of randomly generated Tn insertion mutants is the usage of invasive bacteria as vehicles of virus reconstitution. Certain *Salmonella* strains and *E. coli* strains expressing the bacterial gene invasin and listeriolysin can invade mammalian cells and release plasmids. Experimental transfer of a engineered plasmid-encoded transcription units by invasive bacteria to mammalian cells has been shown both in vitro and in vivo (Darji et al. 1997; Grillot-Courvalin et al. 1998). Accordingly, the MCMV-BAC was

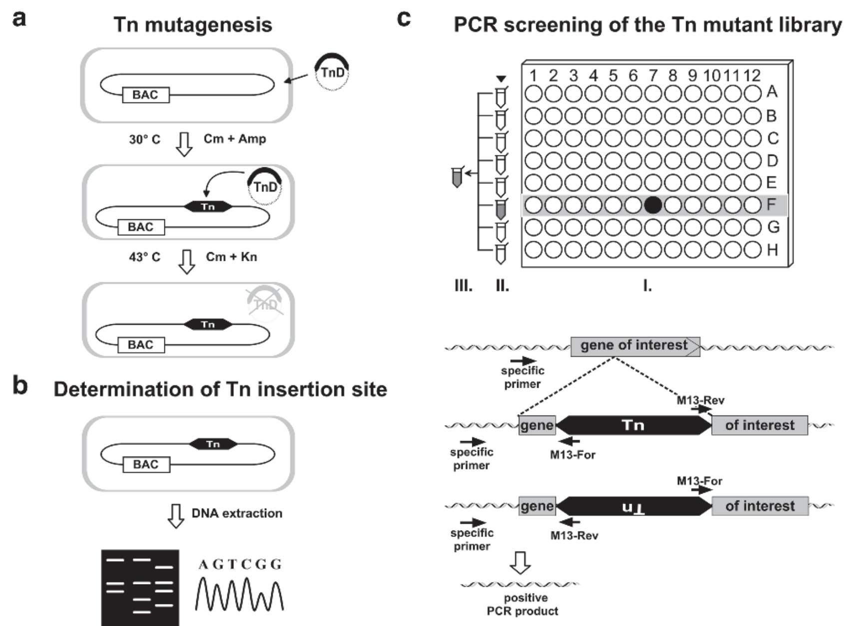


**Fig. 4** Random transposon mutagenesis of herpesvirus BACs and screening for the transposon insertion site. **a** Transposon mutagenesis. A temperature-sensitive transposon donor plasmid (TnD) is transformed into *E. coli* carrying the herpesvirus BAC. If a plasmid-selective transposon is used, transposition leads to insertion of the transposon (*Tn*) into the BAC under antibiotic selection for chloramphenicol (*Cm*) and ampicillin (*Amp*) at the permissive temperature (30°C). By following selection with kanamycin (*Kn*) for the transposon and Cm for the BAC at a nonpermissive temperature (43°C), the donor plasmid gets lost and for transposon-inserted BACs can be selected. **b** Determination of the transposon insertion site. From individual *E. coli* clones carrying a viral BAC with Tn insertion, DNA can be extracted and used for sequencing. Forward and reverse M13 primer binding sites at both ends of the transposon allow sequencing from the Tn into the viral sequence, thereby determining the exact nucleotide position of transposon insertion. **c** PCR screening of the transposon mutant library contained in several 96-well plates. Eight rows (A-H) of 12 individual *E. coli* clones (1-12) from each 96-well plate (*I*) are pooled together into eight single vials (*II*). These eight vials are then pooled into one master vial (*III*) containing 96 different *E. coli* clones with mutant BAC DNA. DNA extracted from individual master vials is used for the PCR screening reaction. Here one specific primer binding up- or downstream to the viral gene of interest and the M13 forward (*M13-for*) and reverse (*M13-Rev*) primers binding to the ends of the transposon are used. A clone with a Tn insertion within the gene of interest gives a positive PCR product generated by the specific primer with one of the M13 primers. The master vial with the positive PCR product is selected and the corresponding eight vials are tested using the same primers. After identification of the corresponding positive vial, all 12 individual *E. coli* clones from this vial are tested and the clone with the transposon insertion within the gene of interest is identified (*black circle*)

released under invasive conditions in host cells, leading to virus reconstitution (Brune et al. 2001b). This procedure allows reconstitution of hundreds of random mutant viruses, thereby setting up direct screens for specific phenotypes and selects for nonessential genes because genomes in which Tns disable essential genes do

not give rise to progeny (Brune et al. 2001b; Menard et al. 2003; Zimmermann et al. 2005). In its random approach, it is most comparable to the classical herpesvirus genetics by chemical mutagenesis. The advantage over the older method is the immediate access to the mutated position. However, Tn mutants identify nonessential gene functions, whereas *ts* mutants characterize essential genes. Interestingly, *E. coli* carrying the infectious herpesvirus BAC can also reconstitute the infectious virus in the natural host. However, only high bacterial load and the parenteral route gave rise to a barely detectable MCMV progeny in vivo (Cicin-Sain et al. 2003).

## *Mutants, Revertants and the Mutation-Phenotype Connection*

The experimental mutations but also spontaneous mutations elsewhere in the genome - or both - may characterize a phenotype. To date, the size of herpesvirus genomes precludes sequencing as a method to completely exclude the contribution of unwanted mutations. Restriction pattern and Southern blot analysis can exclude only gross genomic alterations. Therefore, different experimental approaches have been developed to confirm the observed mutation/phenotype correlation (Fig. 5).

The local genetic change is considered as the causative principle when at least two independent mutants are evaluated and resulted in the same phenotype. The same conclusion is drawn when a revertant is associated with the *wt* phenotype. Both controls support the direct correlation between the targeted site and the observed phenotype. In the *ts* mutagenesis, independent mutants of the same locus confirm the mutation-phenotype connection. CMV-BAC mutants permit both construction of independent mutants of the same locus and generation of a revertant to the *wt* sequence. Beside the shuttle plasmid method, the recent developments in linear fragment mutagenesis provide convenient access to both kinds of controls.

The genotype-phenotype connection, however, cannot answer the question of whether the phenotype is caused by the mutation of the intended gene of interest, or is a consequence of polar effects on other genetic features. Traditionally, a linkage between a specific mutation and a gene product is proven when the principle of the *wt* phenotype can be restored by providing the wt gene product in trans. *Trans*-complementation of CMV gene products is a plausible approach but is associated with major technical difficulties because of the slow virus growth and the limited access of suitable cell lines for genetic engineering. Toxicity of the viral proteins will also inhibit generation *trans*-complementing cell lines.

Recently, a *trans*-complementation of an HCMV gene product by adenovirus mediated transient transduction has been reported which provides a promising alternative approach to deliver CMV genes to infected cells (Murphy et al. 2000).

*Cis*-complementation via reinserting the deleted gene product at an ectopic position into the viral genome is another option (Borst et al. 2001). An FRT site is inserted at a position into the genome where it does not affect the *wt* properties. This *wt*-like viral BAC is used for mutagenesis and the induced phenotypes can be analyzed and

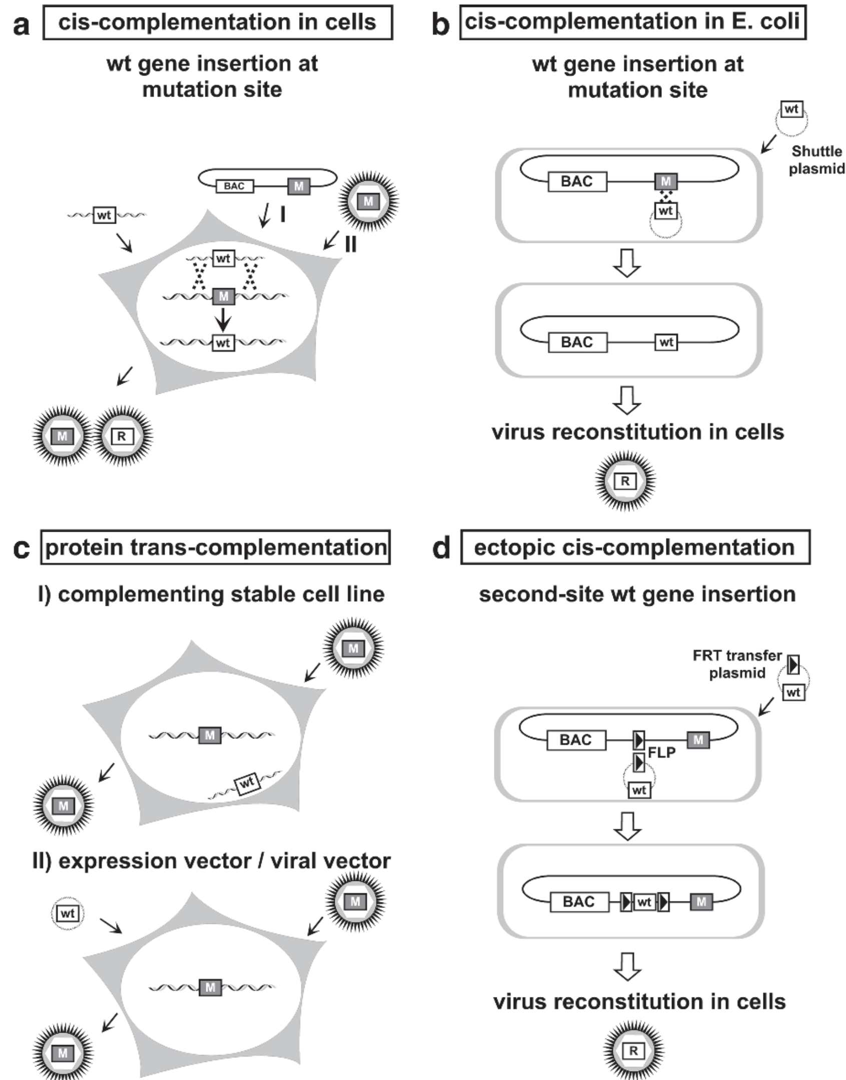


**Fig. 5** Different approaches for confirmation of the mutation-phenotype connection. **a** *Cis*-complementation in cells allows reversion of the mutation to the wt sequence. By transfection of cells with the mutant BAC genome (*I*) or infection with the mutant virus (*II*) and co-transfection of a DNA fragment carrying the wild type (wt) sequence and appropriate viral homologies, the mutation (*M*) can be reverted to the wt sequence. Since revertant and mutant viruses need further separation, this approach only works efficiently if one can select for the revertant, e.g., if it has a growth advantage over the mutant virus. **b** *Cis*-complementation of viral BACs in *E. coli* is best performed by shuttle plasmid mutagenesis. The shuttle plasmid carrying the wt sequence and appropriate homologies is introduced into *E. coli* carrying the mutant BAC plasmid. By RecA-mediated homologous recombination, the wt sequence is inserted at the mutation site without leaving any operational sequences. After transfection of the revertant BAC genome into permissive cells, a homogenous revertant population is gained without any further need for selection against mutant viruses. **c** Protein *trans*-complementation in cells. Cells that express the viral wt gene product permanently (*I*) or transiently by an additional expression vector (*II*) are superinfected with the mutant virus. This allows transient complementation of the mutant phenotype if the expression times and levels of the wt gene product are appropriate. **d** Ectopic *cis*-complementation using viral BACs.

compared to *wt*. To generate the phenotypic reversion, first a rescue plasmid is generated by cloning the *wt* gene into an FRT transfer plasmid that carries an FRT site and can only be maintained in special *E. coli* strain by a conditional origin of replication. This rescue plasmid is then reinserted into the mutant genome using the FRT/Flp system. In contrast to the commonly used approaches, in which the FRT sites are located on the same DNA molecule and mediate deletions or inversions, here the FRT sites induce intermolecular recombination, resulting in the unification of the mutant BAC and the rescue plasmid. The recombinants are selected by the antibiotic resistance of the rescue plasmid and the recombinants are reconstituted for analysis of the phenotype. Ectopic reinsertion, like *trans*-complementation, will not restore polar effects at the site of mutation. However, this approach has advantages compared to protein *trans*-complementation by cells: (a) cell toxicity of the viral gene product is not an issue; (b) the gene expression is controlled by the virus life cycle; (c) Complementation works in any cell-type since the complementing gene is expressed from the virus and permits the analysis of in vivo phenotypes; and (d) it does not require cumbersome establishment of *trans*-complementing cell lines. Ectopic *cis*-complementation system has been established in the context of both MCMV and HCMV and was proven for essential and nonessential genes in vitro and in vivo (Borst et al. 2001; Bubeck et al. 2004; Bubic et al. 2004).

## Genetic Analysis of Essential Genes

### *Comprehensive Mutational Analysis of Essential CMV Genes*

Detailed genetic analysis of genes involved in DNA replication and packaging, morphogenesis and egress of infectious particles, requires expression of mutant genes in the viral context, where the relevant functions are expressed in operational conditions. High-resolution genetic analysis has been demonstrated by elegant pool screens for genetic foot-printing of viral genes, subgenomic fragments, and even of complete viral genomes up to 10 kbp in size (Laurent et al. 2000; Rothenberg et al. 2001). These screens discriminate between virus mutants that replicate and mutants in which essential functions are affected and therefore cannot be retrieved. The transfection of viral DNA corresponding to the size of a CMV genome is not yet efficient enough for pool reconstitution. Therefore, CMV

**Fig. 5** (continued) The wt gene can be introduced into the mutant viral BAC at a neutral second site. By FLP-mediated site-specific recombination of an FRT transfer plasmid carrying the wt gene including regulatory sequences and the mutant BAC genome with an FRT site (*black triangle in a square*), the wt gene product can be expressed from the mutant genome itself. After transfection of permissive cells with this revertant BAC genome, a homogenous population of revertant virus is reconstituted. Only protein *trans*-complementation (**c**) and ectopic *cis*-complementation (**d**) allow the formal confirmation that the mutated gene product (and not possible other *cis*-effects of the mutated sequence) is responsible for the observed phenotype

mutants need to be analyzed individually. The random transposon mutagenesis of the BACs targets the entire genome and null mutants of the respective genes discriminate essential from nonessential genes. Yet, these insertion libraries cannot be used for the functional characterization of a coding sequence. Comprehensive mutant pools of subcloned genes can be obtained through different random mutagenesis procedures. However, a large set of these mutants has to be introduced one by one into the CMV genome lacking the gene of interest to analyze their effect in the context of virus replication. Therefore, we developed a strategy combining a comprehensive Tn7-based linker-scanning mutagenesis of isolated genes (Biery et al. 2000) with fast reinsertion of mutants at an ectopic position into the viral genome by FLP/FRT-mediated site-specific recombination (Fig. 6) as described

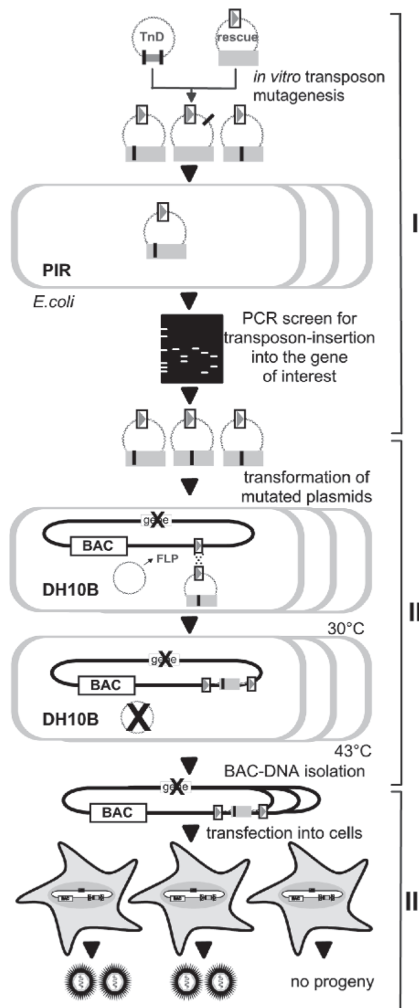


**Fig. 6** Scheme of the strategy for random mutagenesis of an essential viral gene in the viral genome context. Part I: In the first step, the viral gene of interest (*gray box*) is subcloned into a rescue plasmid (*rescue*) containing one FRT site (*open box with gray triangle*) This plasmid is subjected to an in vitro Tn7-based random mutagenesis procedure, leading to a mutant library with 15-bp insertions (*black box*) through the target plasmid. This mutant library is transformed into special *E. coli* strain (PIR) that is permissive for the rescue plasmid and single clones are screened by PCR or followed by sequencing to identify insertions within the ORF under study. Part II: To reinsert the gene mutants into the viral genome lacking the gene of interest, the respective deletion mutant-BAC and a FLP recombinase-expressing plasmid (FLP) are maintained in normal *E. coli* strain (DH10B) and transformed with the rescue plasmids. FLP recombinase mediates site-specific recombination between the FRT sites and unifies the BAC and the rescue plasmid. Combined selection identifies the recombinant BACs with the inserted rescue plasmid because the rescue plasmid itself cannot be maintained in normal *E. coli*. The FLP-expressing helper plasmid is removed by elevated temperature. Part III: Subsequently, BAC DNAs are isolated and transfected one by one into eukaryotic cells for virus reconstitution and cells are screened for viral plaques

above for ectopic *cis*-complementation (see Sect. 4.6). The *wt* gene is subcloned in an FRT containing rescue plasmid. This construct is subjected to random Tn mutagenesis, as described for small plasmids. Mutants are sequenced and a comprehensive set of mutants is selected. Recombination between the FRT transfer plasmids carrying the mutants and the BAC lacking the gene of interest, mediated by the FLP recombinase, provides genomes that are *cis*-complemented by the mutant set. The complemented genomes are tested one by one for virus rescue. In combination with standard biochemical or cell biological assays, this procedure allowed genetic analysis of essential gene functions of MCMV at high resolution (Bubeck et al. 2004; Lotzerich et al. 2006). The method easily maps functionally important sites in essential viral proteins.

## *Identification and Analysis of Dominant Negative Mutants*

The function of nonessential genes is studied by gene deletion and by loss-of-function mutants. By *cis*-complementation assays functionally important sites of essential genes can be mapped. Unfortunately, a major target of genetics, the null phenotype of an essential gene is generally hard to come by. This requires the cumbersome establishment and optimization of a *trans*-complementation system for each gene under study. Here, we try to develop a systematic approach. Dominant negative (DN) mutants are special null mutants that induce the null phenotype even in the presence of the *wt* allele. DN mutants of cellular genes have been proven to be a valuable for genetic analysis of complex pathways (Herskowitz 1987). Knowledge of protein structure, protein functions or sequence motifs aid the design of DN mutants (Crowder and Kirkegaard 2005). Unfortunately, the information on the majority of herpesvirus proteins is too limited for knowledge based construction of DN mutants.

Therefore, we set up a random approach to isolate DN mutants of CMV genes. The Tn7-based linker-scanning mutagenesis introduces 5-aa insertions into coding sequences and provides a comprehensive set of subtle insertion mutants of the ORF. Nonfunctional mutants are selected from a library by a *cis*-complementation screen as described above. These mutants can then be introduced into the *wt* MCMV genome. This allows testing their inhibitory potential (Fig. 7a). If the mutant interferes with the function of the essential *wt* allele, virus reconstitution is inhibited. As we showed for both M50 (Rupp et al. 2007) and the M53 (Z. Ruzsics and U.H. Koszinowski, unpublished data), such mutants represent only a small proportion of the null mutants but can be isolated by a standardized procedure.

Transfection of the viral nucleic acid is error-prone and not only a DN function, but also unrelated effects may prevent virus reconstitution. Conditional expression of the inhibitory mutants in the context of the *wt* genome should allow virus reconstitution in the off state and should induce the null phenotype when turned on (Fig. 7b). We constructed a regulated expression system for MCMV (Rupp et al. 2005) in which the constitutive viral expression of the TetR blocks the transcription of the

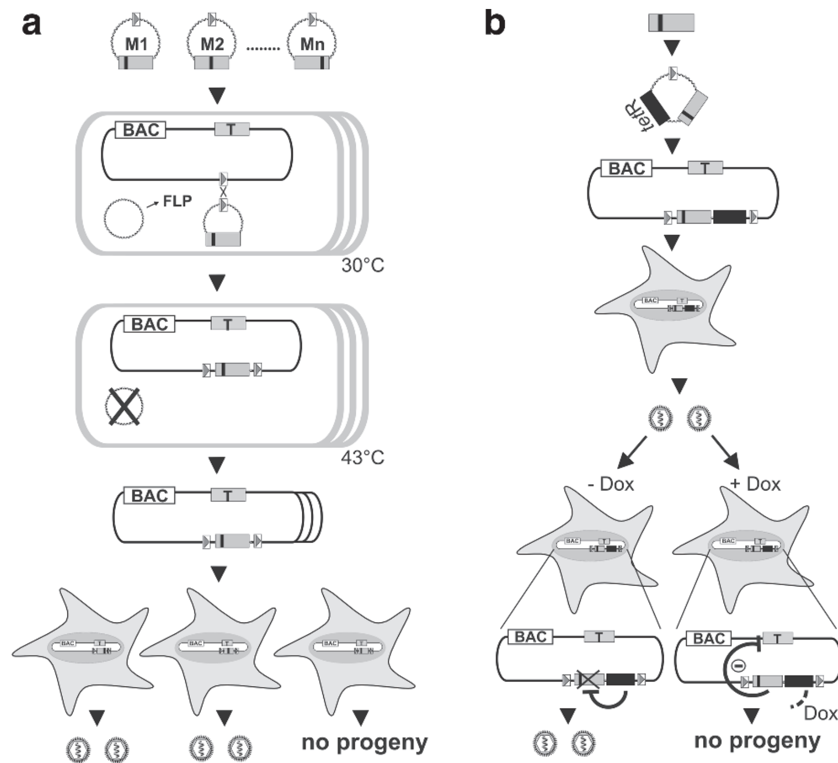


**Fig. 7** Screening for and characterization of dominant-negative mutants of essential viral genes. **a** Screening for inhibitory mutants. An essential viral gene, the target gene (*gray box, T*), is subcloned and subjected to a random and comprehensive mutagenesis in vitro leading to a mutant library M1, M2, . . . Mn (*small black boxes* indicate mutations). Mutated ORFs are placed under the control of a strong constitutive promoter into an insertion plasmid containing an FRT site (*open box with gray triangle*). The insertion plasmids can only be maintained in a special *E. coli* strain. Normal *E. coli* (*open boxes*) carrying an FRT site-labeled viral bacterial artificial chromosome (*BAC*) and a temperature-sensitive plasmid-expressing FLP recombinase (*FLP*) are transformed with the insertion plasmids carrying different mutants one by one. The FLP recombinase mediates site-specific recombination between the FRT sites in the BAC and the insertion plasmids. This recombinants can then be isolated under combined antibiotic selection for both the BACs and the insertion plasmid. The FLP-expressing helper plasmid is removed by elevated temperature. Then BAC DNA is prepared and permissive cells are transfected with each construct. The mutants that are able to inhibit the virus reconstitution can be selected on the basis of the inability of plaque formation upon transfection. **b** Validation of dominant negative mutants by conditional gene expression. The inhibitory mutants are subcloned under the control of a promoter regulated by the TetR (*black box*) into an insertion plasmid with an FRT site. These constructs are delivered into the viral BAC as described above. Then permissive cells are transfected with the recombinants in order to reconstitute viruses carrying the regulation cassettes for the inhibitory mutants. The inhibitory mutants are not expressed during reconstitution because in the absence of doxycycline (- *Dox*), the constitutively expressed TetR blocks their transcription. The inhibitory function of the mutants can be analyzed upon doxycycline administration (+ *Dox*), which leads to the expression of the inhibitory mutant by releasing the expression cassette from the TetR regulation

regulated gene and induction by doxycycline exposes the viral replication program to the DN mutant. This system allowed us detailed quantitative and qualitative analysis of the effect of DN mutants of both M50 and M53. In addition, the result of the random screen on MCMV M50 aided the construction of a DN mutant of the homolog in HCMV (UL50) (Rupp et al. 2007). We believe that this systematic approach will facilitate the functional analysis of essential CMV genes.

## Concluding Remarks

Cloning large DNA sequences as BACs has become the method of choice for mapping, sequencing and manipulation of large eukaryotic genomes. Genetic engineering in BACs is based on homologous recombination and now allows any type of DNA modification. In addition, for herpesviruses, including CMV, these procedures permit the manipulation of the infectious genome as a single plasmid. Mutagenesis is safely carried out in *E. coli* and physical controls can be performed prior to virus reconstitution. These are necessary because CMV genomes contain repetitive sequences that are prone to recombination. In the past, the investigation of CMV gene functions was limited by the laborious and time-consuming generation of virus mutants. Using BAC recombineering, this problem is solved. In the future, more weight has to be placed on careful planning of appropriate controls. The lack of technical limitations allows the production of a plethora of mutants. Traceless mutagenesis permits multiple sequential mutagenesis steps and complex engineering procedures. However, only the local targeted mutations can be thoroughly checked. There is an uncertainty on the numbers of unwanted mutations in other regions that may occur. These mutations do not need to have a growth phenotype in the cell line under study but may mar experiments in other cells or in vivo experiments. According to the law of error propagation, the necessary controls increase with each sequential mutagenesis step. Since at the level of plasmids each step can be controlled by sequencing, as many steps as possible should be done on subcloned target regions. With regard to BAC engineering, it may be advisable to decide on a smaller number of BAC engineering steps rather than on perfect sequence correction.

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