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A sequence-independent *in vitro* transposon-based strategy for efficient cloning of genomes of large DNA viruses as bacterial artificial chromosomes

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ABSTRACT

Bacterial artificial chromosomes (BACs) derived from genomes of large DNA viruses are powerful tools for functional delineation of viral genes. Current methods for cloning the genomes of large DNA viruses as BACs require prior knowledge of the viral sequences or the cloning of viral DNA fragments, and are tedious because of the laborious process of multiple plaque purifications, which is not feasible for some fastidious viruses. Here, we describe a novel method for cloning the genomes of large DNA viruses as BACs, which entails direct *in vitro* transposition of viral genomes with a BAC cassette, and subsequent recovery in *Escherichia coli*. Determination of insertion sites and adjacent viral sequences identify the BAC clones for genetic manipulation and functional characterization. Compared to existing methods, this new approach is highly efficient, and does not require any information on viral sequences or cloning of viral DNA fragments, and plaque purifications. This method could potentially be used for discovering previously unidentified viruses.

INTRODUCTION

Large DNA viruses, including herpesviruses, poxviruses, baculoviruses, nudiviruses and ascoviruses, contain large linear or circular double-stranded DNA genomes with sizes over 80 kbp. These viruses affect vertebrates (mammals, birds, reptiles, amphibians and fishes) and invertebrates (insects and mollusks) across the animal kingdom. Genetic mutations of viral genomes are a standard approach for functional delineation of viral genes. The cloning of genomes of large DNA viruses as bacterial artificial chromosomes (BACs) in *Escherichia coli* could greatly facilitate their genetic manipulation (1). The genomes of several large DNA viruses, including human cytomegalovirus, herpes simplex virus type 1, Epstein-Barr virus, Kaposi's sarcoma-associated herpesviruses (KSHV), murine cytomegalovirus, pseudorabies virus, Marek's disease virus, murine gammaherpesvirus-68 (MHV-68), rhesus rhadinovirus, varicella-zoster virus, guinea pig cytomegalovirus and vaccinia virus have been cloned as BACs in the last decade (1–14). These recombinant BACs have become important tools for studying the functions of viral genes of these viruses (15,16).

Currently, the cloning of the genome of a large DNA virus as a BAC is achieved by homologous recombination between the viral genome and a BAC cassette with flanking viral DNA fragments, or alternatively, as overlapping cosmid inserts encompassing the entire viral genome, with one insert containing the BAC vector (15). These methods require some prior knowledge of the viral sequence or the cloning of a viral DNA fragment to generate the BAC cassette or the BAC vector-containing cosmid insert. The process of multiple plaque purifications is tedious and time-consuming even under drug selection, and is further aggravated by the low recombination efficiency in cells and the slow growth nature of some large DNA viruses. For fastidious viruses, such as KSHV that do not produce any plaques in culture, this becomes a daunting task (12). In this study, we have developed a novel method for direct efficient cloning of genomes of large DNA viruses as BACs without any prior knowledge of viral sequences or cloning of viral fragments. We have used MHV-68, a gammaherpesvirus with a 110 kbp

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double-stranded DNA genome, as an example to illustrate the high efficiency, simplicity and rapidness of this method.

MATERIALS AND METHODS

Cell and virus

Both NIH3T12 cells and MHV-68 WUMS strain (ATCC VR1465) were kindly provided by Dr Herbert W. Virgin IV at Washington University School of Medicine. NIH3T12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 50 μg/ml of gentamycin. The growth of MHV-68 in NIH 3T12 cells and virus plaque assay were carried out as previously described (17).

Isolation of viral and BAC DNA

Extraction of viral DNA from virions was carried out as previously described with minor modifications (17). Infected cells were subjected to three cycles of freeze-thaw, and cell debris was removed by low-speed centrifugation at 5000 r.p.m. for 15 min. Cleared supernatant was spun at 10 000*g* for 2 h to pellet the virus. The virus pellet was then resuspended in DNase buffer containing 50 mM Tris–HCl at pH 7.5, 10 mM $MgCl_2$ and 50 μg/ml of BSA. DNase was added and the virus suspension was incubated for 1 h at 37°C to allow digestion of cellular DNA. After centrifugation at 111 000*g* for 1 h with a 20% sucrose cushion, the cellular DNA-free virus in the pellet was resuspended and treated with proteinase K in a lysis buffer containing 20 mM Tris–HCl at pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% sarkosyl and 0.5% SDS. After extraction with phenol/chloroform, the viral DNA was precipitated with isopropanol, washed with 70% ethanol, dissolved in TE containing 10 mM Tris–HCl at pH 7.5 and 1 mM EDTA.

BAC DNA from *E. coli* was isolated as previously described (12). Isolation of episomal viral DNA was performed according to a published method (18).

Construction of Tn5-BAC vectors pHAIME and pHGCME

Plasmid pHAI was kindly provided by Dr Nikolaus Osterrieder at the College of Veterinary Medicine, Cornell University with the permission of Dr Martin Messerle at the Institute for Virology, Hannover, Germany. PacI-linearized pHAI consists of a mini-F sequence, a chloramphenicol (CM) resistance gene and the *E. coli* guanine phosphoribosyltransferase (gpt) selection marker, and is flanked at both ends by 34-bp loxP recombination sites (Figure 1A). An annealed double-stranded oligonucleotide, 5′-pCGAGATGTGTA TAAGAGACAGGCGGCCGC CTGTCTCTTATACA CATCT CGAT-3′, containing AT overhangs at both ends and one NotI site flanked with two Tn5 transposase recognition sequences as shown in underline, was then inserted into the PacI site of pHAI to generate pHAIME by site-specific ligation using T4 DNA ligase (Promega, Madison, WI, USA).

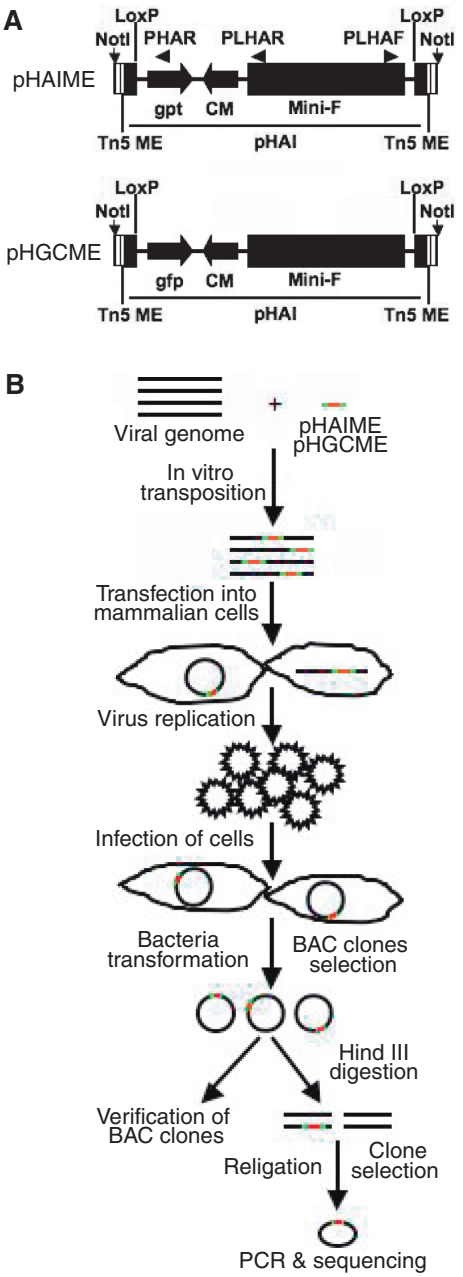

Figure 1. Schematic illustration of the strategy for efficient sequence-independent *in vitro* transposon-mediated cloning of genomes of large DNA viruses as BACs. (**A**) NotI-linearized Tn5-based BAC vector cassettes pHAIME and pHGCME. pHAIME contains 19 bp mosaic end Tn5 transposase recognition sequences (Tn5 ME) on both ends of pHAI, which consists of gpt gene, CM resistance gene, mini-F sequences and flanking loxP sites. PLHAR and PLHAF were designed for long PCR amplification of the junction sequences. PLHAF and PHAR were also used for DNA sequencing. pHGCME is similar to pHAIME except the gpt cassette was replaced with a gfp cassette. (**B**) Procedure for molecular cloning of the genome of a large DNA virus as a BAC by Tn5-mediated direct *in vitro* transposition. An *in vitro* transposon insertion reaction was carried out with viral genomes, EZ-Tn5™ transposase and BAC vector cassette pHAIME or pHGCME. The reaction products were transfected into cells and the resulting progeny viruses were used to infect new cells for isolating viral episomal DNA. Following recovery in *E. coli*, BAC clones were screened by restriction digestion and Southern blot hybridization analysis.

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pHGCME was constructed by replacing the gpt cassette with a green fluorescence protein (gfp) cassette through a two-step recombination process as previously described (12). The gfp cassette was amplified from plasmid pEGFP-M with primers: 5′-AGTAATCAATTACGGG GTC ATTAGTTC-3′ (FCZ17) and 5′-TTTGGA TCCA AGATACATTGATGAGTTTGGAC AA-3′ (FCZ18). The Kan cassette was generated by PCR amplification using EZ-Tn5™ <KAN-2> as a template (Epicentre, Madison, WI, USA) with primers: 5′-CACGTTGTGT CTCA AAATCTCTGATG-3′ (KanF) and 5′-TTTGG ATCCGGTTGATGAGAGCTTTGTTGT AGGTG-3′ (KanR). The PCR products were digested with BamHI and ligated with T4 DNA ligase. The ligated product was used as a template to generate a PCR product using primers: 5′-CGGGCGTATTTTT TGAGTTATCGAGATT TTCAGGAGCTAAGGAAGCTAAAATGAGCCATAT TCAAC GGGAAAC-3′ (KGF) and 5′-GCCCAGCGTC TTGTCATTGGCGAACTCGAACAC GCAGATGCA GTCGGGGCGAGTAATCAATTACGGGGTCATTA GTTC-3′ (KGR) for the first recombination between pHAIME and the PCR product based on the underlined recombination sequences to obtain pHGKME. The Kan cassette was then replaced by a CM cassette, which was amplified from the pHAI plasmid with primers: 5′-CGG GCGTATTTTTTGAGTTATCGAGATTTTCAGG AG CTAAGGAAGCTAAAAT GGAGAAAAAAATCACT GGATATACCACC-3′ (CMF) and 5′-GCATTTTTTTC ACT GCATTCTAGTTGTGGTTTGTCCAAACTCAT CAATGTATCTTACTGCCTTAAAAAAATTACGCCC CG-3′ (CMR). The product was used for the second recombination with pHGKME in *E. coli* strain DH10B based on the underlined recombination sequences, which resulted in the generation of pHGCME.

Identification of transposon integration site

PCR was performed using the indicated templates to amplify the integration junction viral sequences with primers: 5′-CGGTGTGCGGTTGTATGCCTGCTGTGG-3′ (pLHAF) and 5′-GATCCGCGCTTTCTCTGTCCTTC CTGTGC-3′ (pLHAR). The resulting PCR products were sequenced using primers PLHAF and 5′-CGTA AACTCGCAAGCCG-3′ (PHAR), respectively.

In vitro transposition reaction

Two sets of experiments were performed. In the first set, we determined the efficiency of *in vitro* transposition of the MHV-68 genome using the Tn5-BAC vector pHGCME, which contains a gfp cassette to facilitate the monitoring of cells harboring the recombinant viral genomes. Viral DNA at 1, 0.3 and 0.1 μg were used, together with molar ratios of the Tn5-BAC vector and the MHV-68 genome maintained at 3:1, 1:1, 0.3:1 and 0:1, respectively. The transposon reactions contained the linearized pHGCME DNA, the MHV-68 DNA and 1 U of EZ::TN transposase (Epicentre) in 20 μl of EZ::TN reaction buffer. After incubation at 37°C for 2 h, EDTA was added to a final concentration of 10 mM and the mixture was heated for 20 min at 65°C to inactivate the transposase. The efficiencies of transposon integration under different conditions were examined following transfection of the reaction products into the NIH3T12 cells. In the second set of experiments, a transposon reaction containing 1 μg of MHV-68 DNA together with pHAIME and viral DNA at a 1:1 molar ratio was carried out. Individual MHV-68 BAC clones were randomly selected for characterizations from this set of experiments.

Transfection of viral DNA into NIH3T3 cells and observation of viral plaques

Cells at 70–80% confluency were transfected with *in vitro* transposed viral DNA preparations using Targefect-293 F-2 Transfection reagent according to the instructions of the manufacturer (Targeting Systems, El Cajon, CA, USA). To visualize the efficiency of transposon integration into the viral genome, the plates were scanned with a Typhoon™ 9410 Imaging System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) to record the numbers of viral plaques per plate. Individual viral plaques were confirmed with a Zeiss Axiovert 200M Fluorescent Microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA).

Transfection of episomal viral DNA into *E. coli*

Transfection of episomal viral DNA into *E. coli* was carried out as previously described (12).

Restriction analysis and Southern blot hybridization

DNA preparations of recombinant viruses were treated with KpnI restriction enzyme, separated on 0.8% agarose gels by electrophoresis and transferred to Zeta-Probe GT membranes. The membrane was hybridized with a ^{32}P-labeled PHAI probe, and specific signals were detected with the Typhoon™ 9410 Imaging System.

RESULTS

Sequence-independent *in vitro* transposon-mediated cloning of the MHV-68 as infectious BACs

We adapted the Tn5 transposition system from Gram-negative bacteria (19) to insert the BAC vector into the MHV-68 genome. We first generated two Tn5-based BAC vector cassettes, pHAIME and pHGCME as described in Materials and Methods section (Figure 1A). Briefly, a 5′-phosphorylated oligonucleotide containing a NotI site flanked by two Tn5 mosaic sequences was inserted into the PacI site of the pHAI plasmid, which consists of a bacterial origin of replication, a CM cassette and an *E. coli* gpt selection marker flanked at both ends by loxP recombination sites. pHGCME was generated by replacing the gpt cassette with a gfp cassette. We first determined the efficiency of transposon integration into the viral genome by varying the amount of viral DNA and the molar ratio of transposon cassette versus viral DNA. In this set of experiments, we used the pHGCME construct to facilitate the monitoring of viral infection and replication because of the presence of the gfp cassette. *In vitro* transposition reactions containing viral DNA, EZ-Tn5™ transposase and the NotI-linearized pHGCME were carried out as

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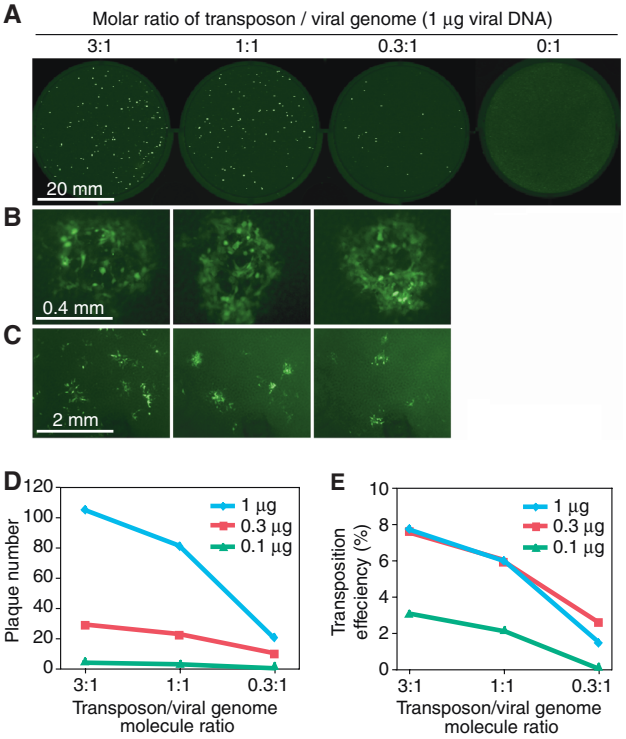

Figure 2. Efficiency of sequence-independent *in vitro* transposon-mediated integration of genomes of large DNA viruses. (**A**) *In vitro* transposition was carried out with 1 μg of MHV-68 DNA under molar ratios of pHGCME and viral genome of 3:1, 1:1, 0.3:1 and 0:1. Following transfection of the reactions into NIH3T12 cells, gfp plaques were observed with a fluorescent scanner. (**B**) Representative individual gfp plaques described in (A) under corresponding molar ratios of pHGCME and viral genome observed under a fluorescence microscope. One plaque was shown for each field. (**C**) Representative gfp plaques after passage of the culture supernatants from (A) to new cultures. The plaques were observed under a fluorescence microscope. Multiple plaques were shown in each field. (**D**) The numbers of plaques per plate obtained under conditions of different viral DNA, and molar ratios of pHGCME and viral genome. (**E**) Efficiency of transposition under conditions of different viral DNA, and molar ratios of pHGCME and viral genome. Transposition efficiency was calculated as the percentage of gfp-positive plaques in total number of plaques per plate.

described in Materials and methods section (Figure 1B). NIH3T3 cells were transfected with the reaction products to reconstitute infectious virus. We used a fluorescence imaging scanner to monitor the formation of viral plaques at day 4 posttransfection. We observed a large number of gfp foci in the range of 80–105 foci per plate when 1 μg of viral DNA was used in combination with 3:1 and 1:1 molar ratios of transposon cassette versus viral DNA (Figure 2A). These foci resembled viral plaques under a fluorescence microscope (Figure 2B), and their sizes grew over time (data not shown), suggesting that they were caused by infectious viruses. Indeed, we observed new plaques following passage of the supernatants to new plates (Figure 2C). When the molar ratio of transposon cassette versus viral DNA was decreased to 0.3:1, the number of plaques was reduced to 20 plaques per plate (Figure 2A), indicating that higher molar ratios of transposon cassette versus viral DNA, preferably at 1:1 or higher, could ensure the transposon integration efficiency. When the viral DNA amount was reduced to 0.3 μg and 0.1 μg, the number of plaques was reduced to 29 and 4 per plate, respectively, even though the transposon and viral DNA ratio was maintained at 3:1 (Figure 2D), indicating that the amount of input viral DNA could significantly influence the number of gfp plaques. We further determined the efficiency of transposon integration into the viral genome by calculating the percentage of gfp-positive plaques in total number of plaques per plate. As shown in Figure 2E, when the 3:1 molar ratio of transposon cassette and viral DNA was used, viral DNA at both 1 μg and 0.3 μg gave similar transposition efficiencies at 7.8% and 7.2%, respectively. While reduction of the transposon cassette and viral DNA ratio also led to the decrease of transposition efficiencies, viral DNA at 1 μg and 0.3 μg remained to have similar transposition efficiencies (Figure 2E). When the viral DNA was decreased to 0.1 μg, the efficiency of transposition into the MHV-68 genome was reduced to 3.0% at the 3:1 transposon cassette and viral DNA molar ratio. As expected, the transposition efficiency decreased proportionally with the reduced transposon cassette and viral DNA molar ratio. These results suggested that higher viral DNA input, preferably at >0.3 μg per reaction, combining with a higher molar ratio of transposon cassette and viral DNA, preferably at >1:1, was required to maintain the transposition efficiency. Furthermore, these results have shown that transposon integration can be achieved with as little as 0.1 μg of viral DNA.

To show the utility of this approach for cloning genomes of large DNA viruses, we further characterized the recombinant viruses cloned by *in vitro* transposon integration. In this set of experiments, an *in vitro* transposition reaction containing 1 μg of viral DNA together with a pHAIME and viral DNA molar ratio of 1:1 was carried out. At day 6 posttransfection, we observed complete cytopathic effect (CPE) in the culture, from which we collected the supernatant containing the progeny virus, and subsequently infected a T-25 flask of NIH3T12 cells. At day 1 postinfection, episomal viral DNA was isolated and transformed into *E. coli* DH10B as described in Materials and Methods section (Figure 1B). Following CM selection, isolated BAC colonies were grown in Luria-Bertani medium for DNA preparation. Restriction analysis was carried out to screen clones that contained the entire viral genome. We obtained 150 clones containing the pHAIME-integrated genomes, from which we randomly selected three clones (clones 1–3) for further analysis.

To determine whether the BAC clones were infectious, we transfected the NIH3T12 cells with the BAC DNA. We observed extensive CPE with all three selected BAC clones at day 5 posttransfection (data not shown). Upon passage of the supernatants to new cultures, we also observed CPE in the new cultures. These results indicated that all three MHV-68 BAC clones were infectious.

Identification of integration sites and adjacent viral sequences

To determine the integration positions of the BAC cassette in the viral genomes in each of the BAC clones, we used

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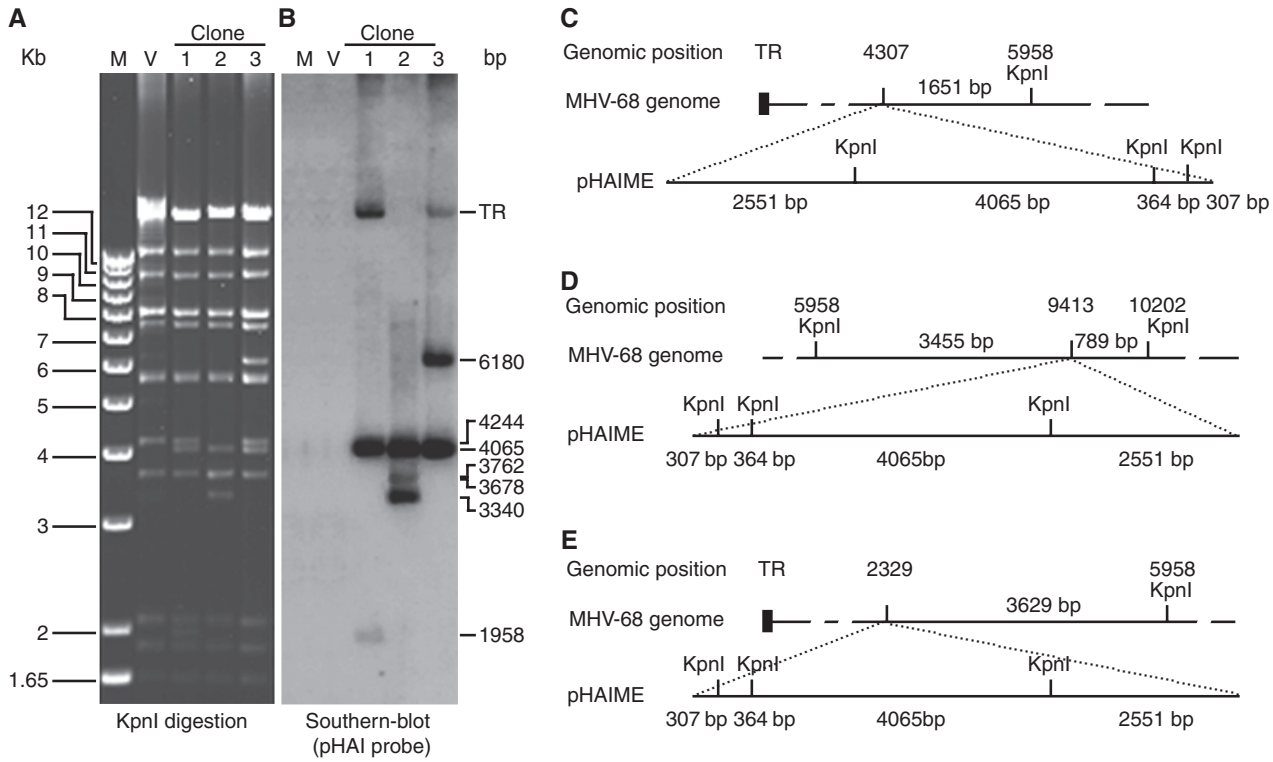

Figure 3. Genetic analysis of MHV-68 BAC clones obtained by sequence-independent *in vitro* transposon-mediated cloning. (**A**) Restriction digestion and (**B**) Southern blot hybridization analysis of selected BAC clones. BAC DNA from clones 1–3 as well as purified wild-type viral DNA (V) were subjected to KpnI restriction analysis (A), and Southern blot hybridization with pHAI vector as a probe (B). (**C–E**) Schematic illustration of recombinant MHV-68 clone 1 (C), clone 2 (D) and clone 3 (E), and the pHAIME cassette with positions of their KpnI sites, cassette integration sites and corresponding DNA fragments before and after integration.

HindIII, which only cuts within the viral genome, to excise the cassette together with the viral sequences flanking the insertion sites (Supplementary Figure 1A). Following religation and transformation into *E. coli*, a new set of pHAIME-containing plasmids was recovered. Primers PLHAR and PLHAF located in the pHAIME cassette (Figure 1A) were used to amplify the products from inside the cassette extending outside to the entire circularized viral sequences in the excised plasmids. We identified independent insertion sites for the three selected BAC clones (Supplementary Figure 1B). DNA sequencing was carried out with primers corresponding to the pHAIME cassette. Sequence alignments of the PCR products with the MHV-68 genome sequence identified the insertion sites of clones 1–3 at nucleotides 4307, 9413 and 2329, respectively, in relative to GenBank accession number U97553.

Genetic analysis of the cloned recombinant viruses

We carried out restriction analysis and Southern blot hybridization with the three selected MHV-68 BAC clones. As shown in Figure 3A, all three clones (1–3) had restriction patterns similar to that of the wild-type viral genome except in bands where the integration of the BAC cassette occurred. The pHAIME cassette contains three KpnI sites generating DNA fragments of 2551 bp, 4065 bp, 364 bp and 307 bp (Figure 3C–E). In clone 1, the transposon cassette was inserted into a large terminal repeat (TR)-containing band at nucleotide 4307 generating a new large TR fragment containing the 2551 bp transposon cassette fragment which is indistinguishable from the original TR band, a new 4065 bp fragment, a new 364 bp fragment and a new 1958 bp fragment derived from the fusion of the 307 bp transposon cassette fragment and a 1651 bp right genomic fragment (Figure 3A and C). The 364 bp band was too small to be visible on the gel. Southern hybridization detected the new TR band, the 4065 bp band and the 1958 bp band (Figure 3B). The 1958 bp band was weak because of limited overlapping sequence (307 bp) with the probe (Figure 3B). In clone 2, the transposon cassette was inserted into the 4244 bp band at nucleotide 9413 generating a new 3762 fragment containing the 3455 bp left genomic fragment and the 307 bp transposon cassette fragment, which is indistinguishable from another 3678 genomic band, a new 364 bp fragment, a new 4065 bp fragment, and a new 3340 bp fragment derived from the fusion of the 2551 bp transposon cassette fragment and a 789 bp right genomic fragment (Figure 3A and D). Again, the 364 bp band was too small to be visible on the gel. Southern hybridization detected the new 3762 bp band, the 4065 bp band and the 3340 bp (Figure 3B). In clone 3, the transposon cassette was again inserted into the large TR-containing band but at nt 2329 generating a new large

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TR fragment containing the 307 bp transposon cassette fragment, which is indistinguishable from the original TR band, a new 364 bp fragment, a new 4065 bp fragment and a new 6180 bp fragment derived from the fusion of the 2551 bp transposon cassette fragment and a 3629 bp right genomic fragment (Figure 3A and E). The 364 bp band was, again, not visible on the gel. Southern hybridization detected the new TR band, the 4065 bp band and the 6180 bp band (Figure 3B). Together, these genetic analyses strongly suggested that all three clones contained the full-length MHV-68 genome.

Since all the large DNA viruses have large genomes and often some repeat sequences, it is essential to ensure that the BAC clones could maintain genetic stability in *E. coli*. Instability of viral genomes in *E. coli* has been observed with herpesviruses (2). We serially propagated all three clones in *E. coli* strain DH10B. Restriction analysis of BAC DNA showed no detectable genomic alteration with all three clones after eight serial passages (data not shown), indicating the stability of the infectious BAC clones in *E. coli*. Thus, under our experimental conditions, we did not detect any instability in the recombinant BAC clones in *E. coli*. Nevertheless, genetic instability might exist for some other clones because of their special integration sites.

MHV-68 ORF-M1 gene is not essential for virus growth *in vitro*

The identification of integration sites of the BAC clones should permit the selection of individual clones that could be used for further genetic analysis of viral genes by targeted mutagenesis or transposon-mediated mutagenesis in *E. coli*. Furthermore, some identified clones with specific insertion disruptions could be directly used for functional characterizations of viral genes. As an example, we selected clone 3 for further characterization. The integration of the BAC vector at nucleotide 2329 should disrupt the ORF-M1 gene, which is encoded from nucleotides 2023 to 3285 of the GenBank accession number U97553. We determined the effect of ORF-M1 disruption on the growth of MHV-68. As shown in Figure 4, the clone 3 virus grew virtually identical to the wild-type virus in both plaque sizes on a cell monolayer and growth curves. These results indicated that ORF-M1 was not essential for MHV-68 replication *in vitro*, which was consistent with results of a previous report (20).

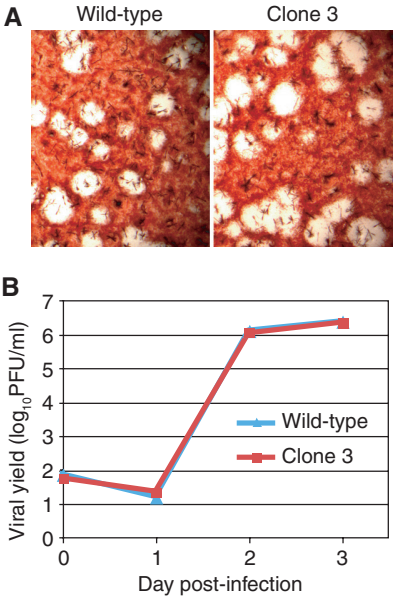

Figure 4. Growth properties of wild-type MHV-68 and BAC clone 3 virus (ORF-M1 mutant). (**A**) Plaque formation of wild-type MHV-68 and BAC clone 3 virus. Viral preparations of wild-type virus and recombinant BAC clone virus from transfected cells (Figure 1B) were subjected to 10 times serial dilution and inoculated on NIH3T12 cell monolayers. Following 1 h adsorption and removal of the viruses, the cells were washed twice with DMEM and overlaid with 0.5% agarose. At day 6 postinoculation, the cells were overlaid with 0.5% agarose containing 0.03% neutral red. Following incubation for 1 day, images of the plaques were captured with a dissection microscope with aid of a digital camera. (**B**) Multistep growth curves of wild-type MHV-68 and BAC clone 3 virus on NIH3T12 cells. The NIH3T12 cells on 6-well plates were infected with wild-type MHV-68 and BAC clone 3 virus at 0.01 multiplicity of infection, respectively. The infected cells were collected at the indicated time points and subjected to three freeze-thaw cycles. After removal of cell debris, the supernatants were used for viral titration.

DISCUSSION

The pioneering work of cloning the genome of murine cytomegalovirus as an infectious BAC has revolutionized the genetics of large DNA viruses (1). Once infectious BACs are obtained, genetic mutations can be introduced into the viral genomes in *E. coli*, and the phenotypes can be assayed upon reconstitution of the recombinant viruses in mammalian cells (15). The genomes of several large DNA viruses have been cloned as BACs by homologous recombination (1–14). These methods require multiple laborious plaque purifications. For viruses, such as KSHV that do not produce any plaques in culture, it is a painstaking process that entails intensive drug-selection and genetic screening (12). Furthermore, these methods require some prior knowledge of the viral sequence or cloning of a viral fragment to generate either the BAC cassette or the BAC vector-containing cosmid insert for homologous recombination. In this study, we have developed a novel method for cloning genomes of large DNA viruses as BACs by combining the highly efficient Tn5-based *in vitro* transposition system (19) with the high capacity of BAC vectors (21). The process is highly efficient and requires as little as 0.1 μg of viral DNA (Figure 2). As a proof-of-concept, we have successfully cloned the MHV-68 genome as BACs, and selected several clones with different integration sites. Using the same method, we have recently cloned the rhesus rhadinovirus genome as BACs (data not shown). As illustrated in the current study, this method is not only efficient but also simple and rapid, and does not require prior knowledge of the viral genome sequences.

The BAC cassette has been shown to interfere with the growth of some viruses (22). Thus, it is desirable to excise the BAC cassette before any functional characterizations. The BAC cassettes used in this study, pHAIME and

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pHGCME, are flanked with the loxP sites, which can be used to excise the cassette (Figure 1A).

Although we have not attempted the experiments, the BAC clones could potentially be recovered in *E. coli* immediately following *in vitro* transposition. Nevertheless, their passages in cell culture could ensure that they were infectious (Figure 1B). Since herpesviruses often contain defective genomes (23), defective BAC clones are commonly seen following BAC cloning reflecting the source of the viral materials (12). While direct cloning of viral genomes might avoid the generation of additional mutations, their passage in the culture could enrich the BAC clones that contain the full-length viral genomes and thus facilitate the initial selection of BAC clones with growth properties resembling the wild-type viruses, which is a prerequisite for their applications in genetic manipulations. Furthermore, recirculation of the viral genomes is required to ensure their growth in *E. coli*. Nevertheless, direct recovery of the cloned genomes into *E. coli* should be useful for circular viral genomes, particularly for viruses that do not have high plaque-forming efficiencies. For large DNA viruses that are close to the genome packaging capacity, such as human cytomegalovirus, direct recovery of the BAC clones in *E. coli* should offer additional advantages.

Herpesviruses can replicate as either linear or circular genome depending on the phase of the viral lifecycle. We have shown that the direct transposon insertion method is highly efficient in cloning linear viral genomes. This strategy should also work for circular viral genomes. However, the sensitivity of this method for cloning circular genomes remains to be determined.

The identification of the BAC cassette integration site can be achieved by direct sequencing of the BAC clone. However, such practice remains a challenge for some sequencing facilities because of the large sizes of the templates. We have used a simple strategy to amplify a PCR product encompassing the integration site, which can then be easily sequenced to determine the integration site of the BAC cassette (Supplementary Figure 1). Such strategy has also been used to map the transposon insertion sites of pseudorabies virus BAC clones (8).

While hundreds of viruses have been identified and their genomes cloned in the last century, many of them remain to be discovered (24). The development of novel methods has often led to the discovery of novel viruses associated with important diseases (25–27). The fact that our method is highly efficient and does not require any prior knowledge of viral sequences renders it useful for discovering previously unidentified viruses. Once the genome of a virus is cloned, its identity can be quickly established following DNA sequencing of the viral fragments adjacent to the BAC cassette integration sites. The BAC clones can then be immediately used for genetic and molecular characterizations of the newly identified viruses.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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