Engineering of a vaccinia virus bacterial artificial chromosome in *Escherichia coli* by bacteriophage λ–based recombination

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The large capacity of vaccinia virus (VAC) for added DNA, cytoplasmic expression and broad host range make it a popular choice for gene delivery, despite the burdensome need for multiple plaque purifications to isolate recombinants. Here we describe how a bacterial artificial chromosome (BAC) containing the entire VAC genome can be engineered in *Escherichia coli* by homologous recombination using bacteriophage λ–encoded enzymes. The engineered VAC genomes can then be used to produce clonally pure recombinant viruses in mammalian cells without the need for plaque purification.

VAC, a member of the poxvirus family, has a linear double-stranded DNA genome of about 200 kilobase pairs (kbp) with covalently closed hairpin ends[1]. Recombinant forms of VAC are used to synthesize biologically active proteins and investigate structure-function relationships, determine the targets of humoral and cell-mediated immunity, and construct live recombinant vaccines[2–4]. The classic technique for inserting foreign genes is based on homologous recombination between a transfer plasmid and the VAC genome in mammalian or avian cells[5]. Typically, multiple cycles of plaque purification are necessary, even with selection or screening protocols. Some advantage has been obtained by the use of purified VAC DNA for *in vitro* or *in vivo* insertion of genes[6–8]. We previously described cloning the full-length VAC genome as a circular BAC[9]. Because poxvirus DNA is not infectious, the VAC-BAC is safe for use with minimal precautions. Infectious virus is readily obtained, however, by transfecting the VAC-BAC into mammalian cells in the presence of a distantly related helper avian poxvirus. The helper fowlpox virus (FPV) provides enzyme functions necessary to initiate replication, but does not produce infectious progeny in mammalian cells and no recombination with the VAC genome is detectable[7]. During rescue and replication, the circular VAC-BAC is linearized and the progeny are indistinguishable from wild-type VAC, except for vector sequences retained in the genome. Our purpose here was to develop methods of genetically engineering the
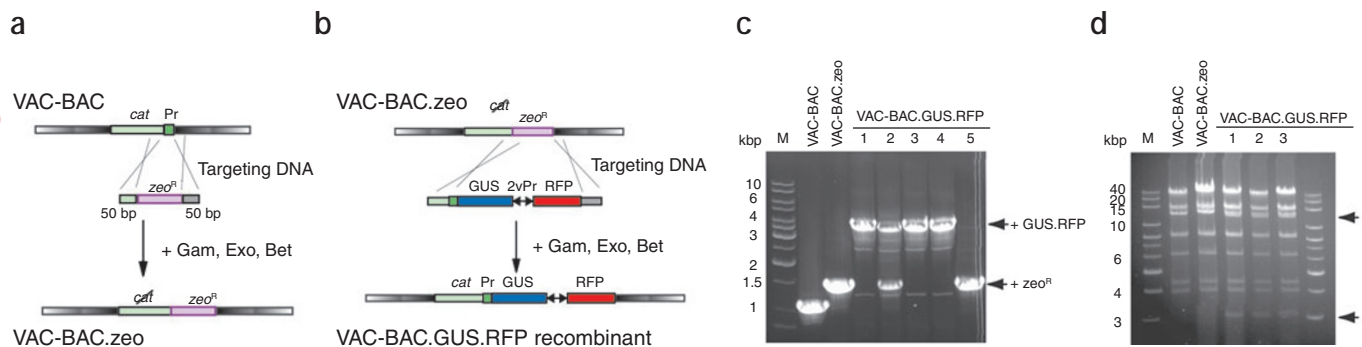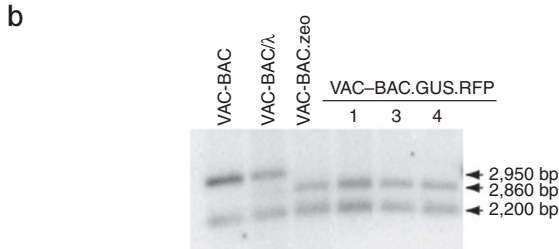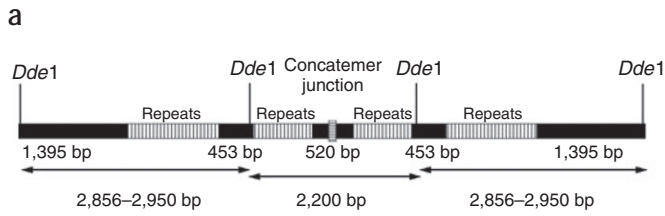

Figure 1 | Engineering VAC-BAC plasmids by homologous recombination. (**a**) Construction of VAC-BAC.zeo. The *red* recombination system in *E. coli* DH10B/VAC-BAC/λ was used to replace the *cat* promoter (Pr) of VAC-BAC with *zeo*R. The targeting DNA contained *zeo*R and 50 bp of flanking DNA excluding the *cat* promoter. Clones were selected for zeomycin resistance and recombination at the correct site was confirmed by PCR and *Hin*dIII restriction endonuclease digestion. Symbol '/', inactivation of *cat* expression. (**b**) Schematic showing insertion of genes encoding GUS and RFP into the VAC-BAC.zeo plasmid to form VAC-BAC.GUS.RFP. The targeting DNA contained RFP and GUS genes adjacent to two VAC promoters (2vPr), the *cat* promoter (Pr) and 371–394 bp of flanking DNA. Bacterial colonies were selected by chloramphenicol resistance. (**c**) PCR screening of VAC-BAC.GUS.RFP plasmids. Five colonies, isolated as in **b**, were analyzed by PCR using 5′ and 3′ primers outside the recombination site. PCR products of the size expected for *zeo* and GUS.RFP are indicated by arrowheads on the right. The GeneRuler 1 kbp DNA ladder (Fermentas Life Sciences) is shown on the left with the sizes of selected bands indicated (M). (**d**) *Hin*dIII restriction endonuclease analysis of VAC-BAC.GUS.RFP plasmid. DNA was extracted from bacteria by alkaline lysis method, ethanol precipitated, washed, digested with *Hin*dIII endonuclease, analyzed by electrophoresis on a 0.7% agarose gel and stained with ethidium bromide. Arrows on the right show the positions of the two predicted additional bands in the VAC-BAC.GUS.RFP plasmid. The 1 kbp DNA extension ladder (Invitrogen) is shown on the left (M) with the sizes of selected bands indicated.

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Figure 2 | Southern blot analysis of the terminal repeat region of VAC-BAC constructs. (**a**) The schematic shows four blocks of 70-bp repeats (striped bars), non-repeat regions (filled bars) and *Dde*I sites in the concatemer junction region of the BAC DNA. The sizes of the non-repeat regions are indicated. Double-headed arrows represent the *Dde*I fragments seen in b. (**b**) Southern blotting with ^{32}P-labeled 70-bp repeat sequence DNA. The original VAC-BAC, VAC-BAC/λ, VAC-BAC.zeo and 3 VAC-BAC.GUS.RFP clones were analyzed.

VAC-BAC to facilitate the production of recombinant VAC expression vectors.

We adapted recombinogenic engineering technology, which avoids the use of restriction enzymes or ligases and allows recombination of BACs maintained in *E. coli*[10,11]. A mini–λ prophage encoding the *red* recombination system, composed of 5′ to 3′ exonuclease (Exo), a single-strand DNA binding protein (Bet) and a nuclease inhibitor (Gam), under the control of the temperature-sensitive λ *c*I857 repressor[12,13], was integrated into the chromosome of *E. coli* DH10B harboring the VAC-BAC to make *E. coli* DH10B/VAC-BAC/λ. The VAC-BAC appeared stable after four successive overnight cultures of *E. coli* DH10B/VAC-BAC/λ and after temperature induction of the λ *red* system for 30–45 min, as judged by restriction endonucle-

ase analysis of DNA from individual clones and by the recovery of infectious VAC following transfection of mammalian cells in the presence of a helper FPV as previously described[9]. Further evidence for stability will be presented below.

The ability of the λ *red* system to mediate homologous recombination was tested by incubating *E. coli* DH10B/VAC-BAC/λ for varying lengths of time at 42 °C to induce expression of the recombination proteins. The cells were then electroporated with linear DNA containing approximately 100 bp of homologous sequences flanking the chloramphenicol acetyltransferase (*cat*) gene essentially as described[13]. The number of chloramphenicol-resistant colonies from cells induced for 0, 15, 30 and 45 min was 0, 7, 47 and 49, respectively. Based on these experiments we recommend the following protocol to induce expression of λ recombination genes. First, 50 ml of LB medium containing tetracycline (25 µg/ml) and chloramphenicol (34 µg/ml) is inoculated with 0.5 ml of an overnight culture of *E. coli* DH10B/VAC-BAC/λ and incubated at 32 °C until the OD$_{600}$ is approximately 0.6. Then, a 10-ml culture is induced at 42 °C for 30 or 45 min in 50-ml flasks. The cells are chilled on ice for 10 min, collected by centrifugation, washed three times with ice-cold 10% glycerol in water and resuspended in 100 ml of the same solution. The cells maintain viability at –80 °C for at least six weeks.

Figure 3 | Formation and analysis of recombinant viruses expressing RFP and GUS. (**a**) Expression of RFP and GUS. CV-1 cells were infected with FPV and transfected with 1 µg of VAC-BAC.RFP plasmid and harvested after 5 d. The lysates were diluted and applied to BS-C-1 cells to allow individual virus plaques to form. After 2 d, the plaques were visualized by fluorescence microscopy with appropriate filters to visualize GFP and RFP. The plates were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid to detect GUS expression. (**b**) *Hin*dIII restriction endonuclease analysis of DNA from VAC-BAC. GUS.RFP virus. Virus from a single plaque was amplified in BS-C-1 cells and purified by sucrose gradient sedimentation. The virus was digested with proteinase K and the DNA was purified by phenol and chloroform extractions and collected by precipitation with ethanol. DNA from VAC-BAC virus and VAC-BAC.GUS.RFP virus was digested with *Hin*dIII restriction endonuclease and analyzed by electrophoresis on a 0.7% agarose gel. The arrow shows the position of the predicted additional band in DNA from VAC-BAC. GUS.RFP virus. The 1-kbp DNA extension ladder is shown on the left (M) with the sizes of selected bands indicated. Note that the second marker band is 20 kbp.

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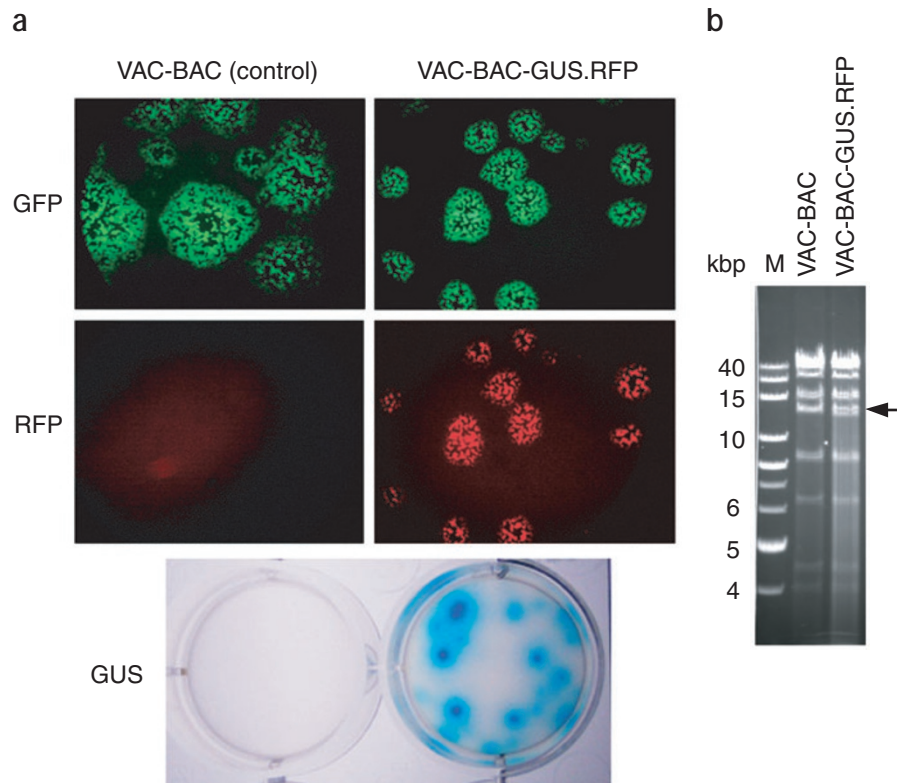
The VAC-BAC is expected to have two main uses: construction of expression vectors and mutagenesis of the VAC genome. The former is of general interest and is the focus of this report. Recombinogenic engineering typically involves two rounds of recombination. In the first round, positive selection is used to isolate clones that have a cassette consisting of adjacent positive and counterselectable genes targeted to the insertion site. In the second round, counterselection is used to isolate clones in which the cassette is replaced with the desired sequence[10]. This approach is extremely versatile, as it can be used for any kind of modification at any place in a BAC. Counterselection is less efficient than positive selection, however, and many colonies must be screened to isolate the final recombinant. To avoid counterselection, we used the *red* recombination system to modify the VAC-BAC for one-step antibiotic selection of recombinants. The promoter of the *cat* gene in the VAC-BAC was replaced by homologous recombination with a zeomycin-resistance gene (*zeo*^{R}) to form VAC-BAC.zeo (**Fig. 1a**). The object of the latter manipulation was to make the *E. coli* sensitive to chloramphenicol, so that subsequent inclusion of the *cat* promoter in an expression cassette would allow isolation of recombinants because of chloramphenicol resistance. In addition to the *cat* promoter, the expression cassette contains the gene of interest regulated by a VAC promoter and flanking sequences comprising the start of the *cat* open reading frame on one side and DNA downstream of *zeo*^{R} on the other. Homologous recombination results in the deletion of *zeo*^{R} and the insertion of the *cat* promoter to restore chloramphenicol resistance and add the gene of interest. To insert a single gene, the insertion cassette is easily built by PCR, as <100 bp of flanking sequence is sufficient for phage-mediated recombination. This would be the preferred method for large-scale projects such as cDNA cloning. For the simultaneous expression of two genes, we constructed a plasmid containing the promoter for the *cat* gene and two VAC promoters with multiple cloning sites between flanking sequences of 300–400 bp (**Fig. 1b**). As a test of the system, the genes encoding glucuronidase (GUS) and red fluorescent protein (RFP) were inserted into the transfer plasmid, each controlled by a separate VAC promoter. The expression cassette was amplified by PCR and the purified product was used for electroporation of *E. coli* DH10B/VAC-BAC.zeo/λ, which had been induced at 42 °C for 30 min. The bacteria were plated on chloramphenicol-containing agar plates and antibiotic-resistant colonies were picked. In the present experiment, four of five colonies examined contained plasmid DNA with sequences encoding GUS and RFP at the targeted site, as determined by PCR using forward and reverse primers (**Fig. 1c**), and one colony was still contaminated with *E. coli* containing VAC-BAC.zeo. Restriction enzyme analysis (**Fig. 1d**) and sequencing of the regions including and flanking the sites of recombination of VAC-BAC.GUS.RFP (data not shown) confirmed the absence of rearrangements, mutations or small deletions accompanying recombination.

To further evaluate the stability of the VAC-BAC after induction of the *red* system, we analyzed the tandem repeat region near the ends of the viral genome. Vaccinia virus contains two blocks of 70 bp repeats separated by a 453-bp unique segment of DNA on each side of the concatemer junction[14] (**Fig. 2**). As a result of recombination, the number of repeats per block naturally varies from 10 to 20 in individual virus isolates[15]. *Dde*I fragments of 2,200 and 2,950 bp were detected by Southern blotting of VAC-BAC DNA

as previously described[14,15], consistent with the presence of two blocks of 12 repeats and two blocks of 16 repeats, respectively (**Fig. 2**). There was no change in the size of either *Dde*I fragment after transformation of *E. coli* with the λ red system, but the size of the larger fragment was reduced by approximately one repeat after insertion of *zeo*^{R} (**Fig. 2**). No further change was noted in any of the three clones encoding GUS and RFP (**Fig. 2**). We attribute the notable DNA stability in *E. coli* DH10B/VAC-BAC/λ to the low copy number of the VAC-BAC and the short induction periods used for homologous recombination.

The final step, rescue of the infectious recombinant virus, was carried out by infecting CV-1 cells with FPV and transfecting them with 1 μg of each VAC-BAC.GUS.RFP plasmid clone as previously described[9]. Cytopathic effects were seen after 3 d at 37 °C and the cells were harvested 2 d later. The recovery of approximately 2×10^5 plaque forming units of infectious recombinant VAC was demonstrated by a plaque assay on BS-C-1 cells, which, like CV-1 cells, are nonpermissive for FPV. All of the plaques contained virus that expressed GUS and RFP as well as GFP, which was present in the original VAC-BAC (**Fig. 3a**). Virus in control plaques expressed only GFP. The presence of the insert was verified by restriction endonuclease digestion of purified VAC-BAC.GUS.RFP viral DNA (**Fig. 3b**).

In summary, recombinogenic engineering of VAC-BAC greatly facilitates the construction and isolation of recombinant virus. Genes can be inserted into the VAC-BAC.zeo plasmid by a one-step procedure with positive selection of bacterial colonies. Moreover, the recombinant VAC-BAC plasmids are then used to produce clonally pure recombinant virus without the need for plaque purifications. The simplified procedure may be particularly useful for high-throughput applications and can be adapted to other BAC vectors.

1. Moss, B. in *Fields Virology* (eds. Knipe, D.M. & Howley, P.M.) 2849–2883 (Lippincott Williams & Wilkins, Philadelphia, 2001).
2. Moss, B. *Proc. Natl. Acad. Sci. USA* **93**, 11341–11348 (1996).
3. Mastrangelo, M.J., Eisenlohr, L.C., Gomella, L. & Lattime, E.C. *J. Clin. Invest.* **105**, 1031–1034 (2000).
4. Paoletti, E. *Proc. Natl. Acad. Sci. USA* **93**, 11349–11353 (1996).
5. Earl, P.L., Moss, B., Wyatt, L.S. & Carroll, M.W. in *Current Protocols in Molecular Biology* (eds. Ausubel, F.M. *et al.*) 16.17.1–16.17.19 (Greene Publishing Associates & Wiley Interscience, New York, 1998).
6. Merchlinsky, M. & Moss, B. *Virology* **190**, 522–526 (1992).
7. Scheiflinger, F., Dorner, F. & Falkner, F.G. *Proc. Natl. Acad. Sci. USA* **89**, 9977–9981 (1992).
8. Smith, E.S. *et al. Nat. Med.* **7**, 967–972 (2001).
9. Domi, A. & Moss, B. *Proc. Natl. Acad. Sci. USA* **99**, 12415–12420 (2002).
10. Copeland, N.G., Jenkins, N.A. & Court, D.L. *Nat. Rev. Genet.* **2**, 769–779 (2001).
11. Britt, W.J., Jarvis, M., Seo, J.Y., Drummond, D. & Nelson, J. *J. Virol.* **78**, 539–543 (2004).
12. Yu, D. *et al. Proc. Natl. Acad. Sci. USA* **97**, 5978–5983 (2000).
13. Court, D.L., Sawitzke, J.A. & Thomason, L.C. *Annu. Rev. Genet.* **36**, 361–388 (2002).
14. Wittek, R. & Moss, B. *Cell* **21**, 277–284 (1980).
15. Moss, B., Winters, E. & Cooper, N. *Proc. Natl. Acad. Sci. USA* **78**, 1614–1618 (1981).