HHi-FiVe: A high-fidelity genetic engineering pipeline for construction of herpesvirus-based vaccines

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Abstract

Herpesvirus-based vectors are attractive for use both as conventional and as transmissible vaccines against emerging zoonoses in hard-to-reach animal populations. However, the threat of off-site mutations during genetic manipulation of vector genomes poses a significant challenge to vaccine construction. Herein, we present the HHi-FiVe (herpesvirus high-fidelity vector) construction pipeline for generating herpesvirus-based vectors by modifying bacterial artificial chromosomes (BACs) and monitoring integrity at each stage by complete genome sequencing. We used this pipeline to repair a highly mutated rhesus cytomegalovirus BAC containing an Ebola virus transgene. The vector derived from this BAC had been shown previously to protect rhesus macaques from lethal Ebola virus challenge by conventional vaccination. Repair of this BAC restored wild-type cellular tropism to the vector, which is essential for transmissible vaccination. Construction of this candidate transmissible vaccine against Ebola virus demonstrates the utility of the HHi-FiVe pipeline for creating precision-made herpesvirus-based vectors.

Introduction

Herpesvirus-based vectors show considerable promise for use as vaccines against infectious diseases. Several such vaccines have been approved for commercial use in agricultural animals, in which they are highly effective. These include a live vaccine based on bovine herpesvirus 1 (Bovilis® IBR Marker Live) to combat infectious bovine rhinotracheitis in cattle and a live recombinant vaccine (VAXXITEK® HVT+IBD) based on turkey herpesvirus to counter infectious bursal disease virus in chickens. Experimental herpesvirus-based vaccines have similarly shown an ability to produce substantial levels of immunity with protection against a range of targeted pathogens, including viruses such as simian immunodeficiency virus and Ebola virus (EBOV), bacteria such as Mycobacterium tuberculosis, and protozoa (Plasmodium knowlesi).

Herpesvirus-based vectors have several key features that have encouraged their development as vaccines. These include an inherently low pathogenic potential, an ability to induce durable levels of antibody-based and T cell-mediated immunity, and a potential for administration via mucosal (i.e. oral and nasal) routes. The vaccines are also amenable to reuse, as prior vector-specific immunity does not prevent reinfection. These features, combined with high host species restriction and the ability to spread among individuals, have motivated the development of transmissible herpesvirus-based vaccines for targeting emerging zoonotic pathogens in the inaccessible wildlife animal populations from which they frequently arise. Advances in bacterial artificial chromosome (BAC)-based genetic engineering have played a large part in the development of technology for manipulating the vectors. Nonetheless, compared to other vaccine modalities, the large genome sizes of herpesviruses and the potential for off-site mutation during manipulation present significant challenges to the widespread use of herpesvirus-based vectors as vaccines, especially in emerging zoonotic disease scenarios, where it is critical to respond rapidly while ensuring the accuracy of vaccine construction.

We have established a robust approach for iterative, high-fidelity genetic engineering of herpesvirus-based vectors. This approach was named the HHi-FiVe (herpesvirus high-fidelity vector) pipeline and was used to restore a total of 13 mutated or missing open-reading frames (ORFs) in a BAC containing a cytomegalovirus (CMV) genome from rhesus CMV (RhCMV) bearing a transgene expressing an EBOV antigen. This BAC (RhCMV68-1/EBOV BAC) was chosen as a starting point because the vector derived from it by transfection has been shown to protect rhesus macaques that were vaccinated subcutaneously and then challenged with normally lethal EBOV doses. As anticipated, the vector reconstituted from the repaired BAC exhibited a phenotype characterized by restored epithelial cell tropism and sustained expression of the transgene (EBOV-GP). This work generated a repaired vector suitable for future model studies of animal-to-animal transmission and demonstrated the practicality of the HHi-FiVe pipeline for producing herpesvirus-based vectors for potential use as vaccines.

Results

RhCMV BACs and ORF nomenclature

Following isolation from the urine of a rhesus macaque in 1968, a parental virus (RhCMV strain 68-1; RhCMV) was subjected to extensive and largely undocumented passage in cultured fibroblasts of human or rhesus macaque origin. A stock of the resulting virus was used to construct a primary BAC (RhCMV BAC) from which all available RhCMV BACs are derived. A succession of
studies has shown that RhCMV$_{68-1}$ and RhCMV$_{68-1}$ BAC are highly mutated$^{18-23}$, with the detail having been revealed progressively by the genome sequences of RhCMV$_{68-1}$, RhCMV$_{68-1}$ BAC, derivatives of RhCMV$_{68-1}$ BAC, viruses generated from RhCMV$_{68-1}$-based BACs, and other RhCMV strains (Table 1). As a result, RhCMV$_{68-1}$ and RhCMV$_{68-1}$ BAC lack the functions of many genes required for cellular tropism and fitness in vivo. It was necessary to repair these mutations in order to create a candidate for testing as a transmissible vaccine. Achieving this involved making multiple small- and large-scale repairs to RhCMV$_{68-1}$/EBOV BAC and carrying out Illumina-based complete genome sequencing at each stage to monitor fidelity.
Table 1
RhCMV genome sequences.

| GenBank accession no. | Parental strain | Source of sequence | Reference |
|-----------------------|-----------------|--------------------|-----------|
| AY186194.1            | 68-1            | Isolated virus     | Hansen et al., 2003^24 |
| JQ795930.1            | 68-1            | BAC                | Malouli et al., 2012^20 |
| MF468139.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018^8  |
| MF468140.1            | 68-1            | BAC                | Hansen et al., 2018    |
| MF468141.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018    |
| MF468142.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018    |
| MF468143.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018    |
| MF468144.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018    |
| MF468145.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018    |
| MF468146.1            | 68-1            | BAC-derived virus  | Hansen et al., 2018    |
| MF468147.1            | 68-1            | BAC                | Hansen et al., 2018    |
| MK937070.1            | 68-1            | BAC                | Marshall et al., 2019^42 |
| MN437483.1            | 68-1            | BAC                | Hansen et al., 2009^4   |
| MT157325.1            | 68-1            | BAC                | Taher et al., 2020^23   |
| MT157326.1            | 68-1            | BAC                | Taher et al., 2020      |
| MT157327.1            | 68-1            | BAC                | Taher et al., 2020      |
| MZ517252.1^b          | 68-1            | BAC                | Present study           |
| MZ517253.1^c          | 68-1            | BAC                | Present study           |
| DQ120516.1            | 180.92          | Isolated virus     | Rivaille et al., 2006^18 |
| KX689267.1            | 19262           | Isolated virus     | Burwitz et al., 2016^21 |
| KX689268.1            | 19936           | Isolated virus     | Burwitz et al., 2016    |
| KX689269.1            | 24514           | Isolated virus     | Burwitz et al., 2016    |
| MT157328.1            | 34844           | Isolated virus     | Taher et al., 2020      |
| MT157329.1            | KF03            | Isolated virus     | Taher et al., 2020      |
| MT157330.1            | UCD52           | Isolated virus     | Taher et al., 2020      |
| MT157331.1            | UCD59           | Isolated virus     | Taher et al., 2020      |
| MZ517254.1^d          | 180.92          | Isolated virus     | Present study           |

^aSequences are listed in order of parental strain and then GenBank accession no.

^bParental RhCMV^68−1^ BAC used in the present study.

^cRhCMV^68−1^/EBOV/RL11G^+^ BAC generated in the present study.

^dFull-length sequence generated in the present study; DQ120516.1 has a large deletion.
The original nomenclature for RhCMV\textsubscript{68−1} ORFs was established in 2003 and consisted of the prefix rh followed by a number (GenBank accession no. AY186194.1)\textsuperscript{24}. This nomenclature was modified and extended in 2012 by comparison with the sequence of RhCMV\textsubscript{68−1} BAC (GenBank accession no. JQ795930.1)\textsuperscript{20}. As this nomenclature related only to RhCMV and not other CMVs, a comparative analysis in 2006 of the sequence of RhCMV strain 180.92 (GenBank accession no. DQ120516.1)\textsuperscript{18} was used to develop a partially inclusive system in which RhCMV ORFs conserved in human CMV (HCMV) were given names corresponding to those in HCMV. A fully inclusive system applying across sequenced primate CMVs was developed in 2011 (GenBank accession no. FJ483968.2)\textsuperscript{22}, when the RhCMV genome annotation was improved further and orthologous ORFs in different CMVs were denoted by the same name. The principal names were those of HCMV ORFs, supplemented by those of ORFs specific to Old World monkey CMVs, which are prefixed by the letter O. This nomenclature is used below and in the genetic map of the final product of the HHi-FiVe pipeline (Figure 1). In addition, when available, the alternative names are provided in Table 2, and the 2012 names are specified below in parentheses after first use of an inclusive name. Nucleotide descriptions are given in relation to the genome sequence regardless of ORF orientation.
Table 2
Steps in repairing inactivated ORFs in RhCMV<sub>68−1</sub>/EBOV BAC.

| Step | ORF | 2003 ORF<sup>a</sup> | 2006 ORF<sup>b</sup> | 2012 ORF<sup>c</sup> | Mutation<sup>d</sup> | Repair<sup>d</sup> | Location<sup>e</sup> |
|------|-----|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| 1    | UL36| rh61&rh60            | rhUL36               | Rh61/Rh60            | Frameshifted in T<sub>8</sub> | Replaced by T<sub>7</sub> | 48669-48675          |
| 2    | UL146C| NP                   | NP                   | NP                   | Wholly deleted       | Replaced              | 167033-171891        |
|      | UL146D| NP                   | NP                   | NP                   | Wholly deleted       | Replaced              |                     |
|      | UL146F| NP                   | NP                   | NP                   | Wholly deleted       | Replaced              |                     |
|      | UL146H| rh161                | NP                   | Rh161                | Partially deleted    | Replaced              |                     |
| 3    | RL11D| rh08                 | rh8                  | Rh08                 | Frameshifted in C<sub>11</sub> | Replaced by CAC<sub>9</sub> | 6341-6350            |
| 4    | UL119| rh152&rh151<sup>f</sup> | rhUL119              | Rh152/Rh151          | Terminated by stop codon (TCA) | Replaced by CCA       | 154761-154763        |
| 5    | US12E| rh197                | rh197                | Rh197                | Terminated by stop codon (CTA) | Replaced by CCA       | 208625-208627        |
| 6    | RL11E| NA                   | rh8.1                | Rh08.1               | Frameshifted in CCAC<sub>10</sub> | Replaced by C<sub>12</sub> | 6991-7002            |
| 7    | RL11B| rh06                 | rh6                  | Rh06                 | Frameshifted in C<sub>11</sub> | Replaced by TCCACCTCC | 5197-5208            |
| 8    | UL128| NP                   | rhUL128              | NP                   | Wholly deleted       | Replaced              | 161705-167032        |
|      | UL130| NA                   | rhUL130              | Rh157.4              | Partially deleted    | Replaced              |                     |
| 9    | RL11G| rh14                 | rh13.1&rh14          | Rh13.1               | Frameshifted due to insertion (CT) | Deleted              | Between 12847 and 12848 |
|      |      |                      |                      | Framedshifted in A<sub>8</sub> | Replaced by A<sub>7</sub> |                      | 13059-13065          |

<sup>a</sup>Name of ORF in RhCMV<sub>68−1</sub>, GenBank accession no. AY186194.1 (Hansen et al., 2003)<sup>24</sup>; NA, not annotated; NP, not present; & separate ORFs; some ORFs are partial because of lack of recognition of errors, mutations or splicing.

<sup>b</sup>Name of ORF in RhCMV strain 180.92, GenBank accession no. DQ120516.1 (Rivailler et al., 2006)<sup>18</sup>; NP, not present; & separate ORFs; some ORFs are partial because of lack of recognition of mutations.

<sup>c</sup>Name of ORF in RhCMV<sub>68−1</sub>BAC, GenBank accession no. JQ795930.1 (Malouli et al., 2012)<sup>20</sup>; NP, not present; /, spliced ORFs; some ORFs are partial because of lack of recognition of mutations.

<sup>d</sup>Sequences correspond to the genome sequence regardless of ORF orientation.

<sup>e</sup>In RhCMV<sub>68−1</sub>/EBOV/RL11G<sup>+</sup> BAC, GenBank accession no. MZ517253.1 (present study).

<sup>f</sup>Apparently intended to be named rh151 but annotated rh141.

**Identification of mutated ORFs in RhCMV<sub>68−1</sub>/EBOV BAC**

As noted previously, one of the complications with identifying mutations in RhCMV<sub>68−1</sub> and RhCMV<sub>68−1</sub>BAC is that the original RhCMV<sub>68−1</sub> sequence appears to contain numerous errors<sup>26</sup>. Thus, some of the differences between RhCMV<sub>68−1</sub> and RhCMV<sub>68−1</sub>BAC are due to these errors rather than to mutations generated during the construction of RhCMV<sub>68−1</sub>BAC. We estimated the number of such errors at 20. They include substitutions and insertions or deletions (indels) in noncoding regions, substitutions in ORFs [RL1 (Rh01), RL11A (Rh05), RL11C (Rh07), RL11D (Rh08) and UL55 (Rh89); and RL11G (Rh13.1)], introducing an in-frame stop codon, although this may have been due to a subpopulation of mutants in RhCMV<sub>68−1</sub> rather than an error, and frameshifts in ORFs [RL11D, COX2 (Rh10), UL34 (Rh57), UL71 (Rh100.1), US18 (Rh199) and US27D (Rh216)].
In order to ensure that the RhCMV component of the repaired RhCMV_{68-1}/EBOV BAC was as close in sequence as possible to the original RhCMV_{68-1} genome as perceived to have existed prior to isolation and serial passage in cell culture\textsuperscript{25}, it was necessary to identify mutations in RhCMV_{68-1} BAC (and hence in RhCMV_{68-1}/EBOV BAC) that have resulted in inactivated ORFs. This involved detailed examination of an alignment of all available RhCMV genome sequences, which at the time did not include several reported since by Taher et al (2020)\textsuperscript{23}; these recent sequences were incorporated at the end of the study and identified no additional mutations. This comparative exercise revealed a total of 13 putatively inactivated ORFs (Table 2). They fell into two categories: (i) those terminated by in-frame stop codons due to substitutions and those truncated or extended by frameshifts due to small indels (most located within or associated with homopolygnucleotide tracts), and (ii) those partly or wholly missing due to large deletions or rearrangements. Seven ORFs [RL11B (Rh06), RL11D, RL11E (Rh08.1), RL11G, UL36 (Rh61/Rh60), UL119 (Rh152/Rh151) and US12E (Rh197)] distributed across the genome were in category (i) and required small-scale repair. Six ORFs [UL128, UL130 (Rh154.7), UL146C, UL146D, UL146F and UL146H] located within a region of the genome called UL/b’, which contains ORFs involved in cellular tropism and immunomodulation\textsuperscript{19,26}, were in category (ii) and required large-scale repair. These ORFs were supplemented by six other ORFs in UL/b’ [UL131A (Rh157.6), UL132 (Rh160), UL148 (Rh159), UL147A, UL147 (Rh158) and UL146B (Rh158.1)] that, although intact and therefore probably not inactivated, were inverted as a block. These 19 ORFs were targeted for repair, replacement, or restoration in RhCMV_{68-1}/EBOV BAC.

Examination of the sequence alignment also indicated a few additional differences in six RhCMV_{68-1} BAC ORFs [O3, UL41A (Rh67.1), UL45 (Rh72), UL74A, UL141 (Rh164) and US12B (Rh194)] that are not represented in RhCMV_{68-1} or other RhCMV strains but caused predicted amino acid substitutions. Given the error-prone nature of the RhCMV_{68-1} sequence, the reality of these differences was not certain, and they were not targeted for repair.

**Pipeline for repairing mutated ORFs**

Targeted genetic manipulation of herpesvirus genomes is achieved by BAC-based recombineering followed by reconstitution of virus by transfection of BACs into permissive cells\textsuperscript{27}. Off-site mutations are a concern when manipulating such large DNA constructs and reconstituting viruses. In the past, this problem has been addressed by creating viruses from revertant BACs in order to demonstrate that the intended manipulations are genetically and phenotypically reversible. However, this approach is regarded as inadequate because it does not control for off-site mutations that arise during reconstitution of virus; in our experience, this is often when such mutations occur. It is also not practical for vaccine development because of the labor-intensiveness and limited scope of phenotypic assays. To cope with this inherent vulnerability, we coupled BAC-based recombineering with responsive Illumina-based whole genome sequencing to create the HHi-FiVe pipeline for generating and validating BACs and reconstituted viruses (Figure 2).

We set out to use this pipeline to repair the mutations in RhCMV_{68-1}/EBOV BAC using BAC-based recombineering\textsuperscript{4,28,29}. Recombinant BACs were screened initially by restriction fragment length polymorphism (RFLP) analysis to screen for appropriate changes to fragment mobility (Supplementary Figure 1). This was followed by whole genome sequencing of recombinant BACs at each stage. Overall, the complete process was accomplished in nine steps (Table 2).

**Small-scale repairs**

Six inactivated ORFs (RL11B, RL11D, RL11E, UL36, UL119 and US12E) required small-scale repair (Steps 1 and 3–7). Most mutations were addressed by restoring the perceived original sequence to reinstate the integrity of the ORF. However, an initial attempt at repairing RL11B at Step 7, which consisted of removing two C residues in a C\textsubscript{11} homopolygnucleotide tract to restore a C\textsubscript{9} tract, resulted consistently in a C\textsubscript{10} tract. Therefore, an alternative strategy was used that involved introducing synonymous substitutions within the tract. Repair of RL11G was also small-scale (see below).

**Large-scale repairs**

A total of 12 ORFs in UL/b’ had undergone extensive deletion or rearrangement during passage of RhCMV_{68-1}, and the six ORFs that were completely or partially missing as a result were not amenable to small-scale repair. Instead, the whole region was replaced by a wild type version based on RhCMV strain 19936 (Table 1), using three synthetic DNA segments that together encompassed this region (Steps 2 and 8). The product of Step 8, which still contained two frameshift mutations in RL11G (see below), was denoted RhCMV_{68-1}/EBOV/RL11G\textsuperscript{−} BAC.

**Repair of RL11G**
RL11G contained two separate mutations: a CT insertion in a (CT)$_2$ tract, and further downstream, an A insertion in an A$_7$ tract. Each mutation resulted in a frameshift, the first removing the transmembrane domain of the encoded protein and the second restoring the correct reading frame near the end of the ORF. The first mutation was predicted to have been sufficient to inactivate RL11G on its own. RL11G is an orthologue of HCMV RL13$^{20}$, which has been shown to mutate during viral growth in culture in all cell types tested$^{30,31}$. Therefore, its repair was reserved for the final step (Step 9). This strategy was vindicated by the recent demonstration that a repaired version of RL11G in a BAC-derived version of RhCMV$_{68-1}$ mutants in rhesus fibroblast culture$^{23}$. Our approach was to delete RL11G completely and insert a full-length synthetic version in which the two frameshift mutations were repaired and two substitutions unique to RhCMV$_{68-1}$ and RhCMV$_{68-1}$-based BACs were replaced. The final product was denoted RhCMV$_{68-1}$/EBOV/RL11G$^+$ BAC and was repaired in all the genes inactivated in RhCMV$_{68-1}$ BAC and RhCMV$_{68-1}$/EBOV BAC by premature termination, frameshifting, deletion, or rearrangement. As well as the intended manipulations and repairs, both RhCMV$_{68-1}$/EBOV/RL11G$^-$ and RhCMV$_{68-1}$/EBOV/RL11G$^+$ BAC had one inconsequential difference from RhCMV$_{68-1}$/EBOV: an additional G residue in a G$_7$-tract in one copy of the terminal direct repeat of the viral genome.

**Stability of RhCMV$_{68-1}$/EBOV/RL11G$^-$ and RhCMV$_{68-1}$/EBOV/RL11G$^+$**

Viruses were reconstituted by transfecting RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC or RhCMV$_{68-1}$/EBOV/RL11G$^+$ BAC into rhesus fibroblast (Telo-RF) or human epithelial (hTERT RPE-1) cells and passaging further. When the cultures exhibited full cytopathic effect, DNA was extracted from infected cells or infected cell supernatant and sequenced. For each dataset, mutations were identified by visual inspection of an alignment of sequence reads to the anticipated viral genome sequence, and their abundance was calculated by counting the proportion of reads containing the mutation. This approach allowed mutations representing major subpopulations to be identified and quantified, and permitted the prevalence of these mutations to be examined in other samples in the same passage series even if present in minor subpopulations. However, minor subpopulations that did not reach sufficient representation in any sample in the series might not have been detected. Three clones (clones 1–3) of RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC and one clone (clone 1) of RhCMV$_{68-1}$/EBOV/RL11G$^+$ BAC were transfected, the latter having been derived from one of the former RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC clones (clone 2). The sequences of the RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC clones were identical to each other. The scheme for reconstituting and passaging viruses is summarised in Figure 3 and the 16 samples sequenced (Samples A–P) are indicated by red font.

Reconstitution of RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC clone 1 in Telo-RF cells generated a 1 bp frameshifting deletion in UL128 and a 193 bp frameshifting deletion in UL116 (Rh148). At passages 1, 4 and 8 in Telo-RF cells (Samples A–C), the proportions of the UL128 mutation were 85, 99 and 100 %, respectively, and the proportions of the UL116 mutation were 4, 0 and 0 %, respectively. Virus at passage 1 in Telo-RF cells was also transferred to hTERT RPE-1 cells. At passages 2 and 5 in these cells (Samples D–E), the proportions of the UL128 mutation were 40 and 30 %, respectively, and the proportions of the UL116 mutation were 38 and 66 %, respectively. Thus, both mutations were present in passage 1 in Telo-RF cells, and the UL128 mutation was selected for in Telo-RF cells but selected against in hTERT RPE-1 cells. In contrast, whereas the UL116 mutation was selected against in Telo-RF cells, it was selected for in hTERT RPE-1 cells. Reconstitution of RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC clone 2 in Telo-RF cells generated a 1022 bp deletion truncating UL128 and UL130 and nine linked C to T substitutions in US12 (Rh190). Among the substitutions, four were synonymous, three were nonsynonymous, and two introduced in-frame stop codons. At passage 1 in Telo-RF cells (Sample F), the percentages of the UL128 and US12 mutations were both 77 %, implying that they were present in the same genome. In contrast, reconstitution of RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC clone 3 in Telo-RF cells generated no major mutations (Sample G). Selection of mutations in one or more of the three adjacent genes UL128, UL130 and UL131A is a recognised feature of RhCMV and HCMV when passaged in fibroblast cells$^{18,30−32}$. Additional mutations may be carried fortuitously with these mutations when present in the same genome, or they may be selected independently. In contrast, UL128, UL130 and UL131A are essential for growth of HCMV and RhCMV in non-fibroblast cells because they encode a glycoprotein complex that is required for viral entry into these cells$^{32,33}$. Consistent with this, no major mutations were generated by reconstitution of RhCMV$_{68-1}$/EBOV/RL11G$^-$ BAC clone 1 in hTERT RPE-1 cells at passages 1, 5 and 10 (Samples H–J), in a mixture of stocks from passages 3–9 in this series (Sample K) grown in hTERT RPE-1 cells, in an independent stock grown from passage 3 in this series grown in hTERT RPE-1 cells, in a mixture of hTERT RPE-1 and Telo-RF cells or in Telo-RF cells alone (Samples L–N), or in an independent stock grown from passage 1 in this series in hTERT RPE-1 cells (Sample O).
In contrast to the results obtained with the RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} clones, reconstitution of RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{+} BAC clone 1 in RPE-1 cells generated a major mutation at passage 1 in these cells consisting of a 12,778 bp sequence extending from within RL1 to close downstream from RL1H that had been replaced by a 1,786 bp bacterial sequence (Sample P). The proportion of genomes in which RL11G had not been inactivated by this indel was close to 0 %. We conclude that virus reconstituted from the RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} BACs was genetically unstable when passaged in Telo-RF cells, accumulating mutations not only in UL128, UL130 and UL131A but also in other parts of the genome. In contrast, the genome was stable when virus was passaged in hTERT RPE-1 cells. Virus reconstituted from RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{+} BAC was unstable in hTERT RPE-1 cells, in which RL11G was inactivated.

**Cellular tropism of RhCMV/EBOV/RL11G\textsuperscript{−}**

The purpose of repairing RhCMV\textsubscript{68−1}/EBOV BAC is eventually to examine its potential as a model transmissible vaccine platform for providing protective immunity against EBOV following animal-to-animal dissemination of the vaccine. The extent to which RL11G is required for dissemination remains to be determined, but the use of virus reconstituted from RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{+} BAC was precluded because of the instability of RL11G in various cell types tested following reconstitution and passage (Figure 3; data not shown), which is consistent with previous findings for RhCMV\textsuperscript{23}, and the RL11G orthologue (RL13) in HCMV\textsuperscript{30,31}. In contrast, the genome integrity of RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} BAC was maintained over multiple passages in hTERT RPE-1 cells. To assess cellular tropism, RhCMV\textsubscript{68−1}/EBOV was reconstituted from RhCMV\textsubscript{68−1}/EBOV BAC in Telo-RF cells, and RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} was reconstituted in hTERT RPE-1 cells. Viral growth was measured in infected Telo-RF cells and hTERT RPE-1 cells. Only RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} was able to replicate in both cell lines (Figure 4).

**EBOV-GP expression by RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−}**

To examine transcription of EBOV-GP, hTERT RPE-1 cells were infected with RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−}, total infected cell RNA was harvested at 21 d p.i., stranded RNAseq data were generated from the polyadenylated RNA fraction, and the relative proportions of sense and antisense RNAs produced from individual coding regions were calculated (Supplementary Table 1). Sense transcripts predominated (93.61 % of all sense and antisense transcripts combined), and EBOV-GP was the sixth most highly expressed (2.31 %) sense RNA of the 185 coding regions assessed. Transcripts from the RL11 family were notable by their generally low level of expression.

Finally, to examine translation of EBOV-GP, Telo-RF cells were infected with RhCMV\textsubscript{68−1} or RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−}, and hTERT RPE-1 cells were infected with RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−}. Immunoblotting was carried out on infected cell proteins using an EBOV-GP-specific monoclonal antibody (mAb) to detect EBOV-GP, a RhCMV UL44 protein-specific antibody to confirm viral infection, and an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAb to monitor cellular protein expression. EBOV-GP was expressed in increasing amounts by RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} in Telo-RF cells at least until 7 d p.i. (Figure 5A) and in hTERT RPE-1 cells at least until 15 d p.i. (Figure 5B).

**Discussion**

This work is a proof-of-concept study aimed at establishing the HHI-FiVe pipeline for efficient, high-fidelity genetic manipulation of herpesvirus-based vectors as a means for providing a rapid turnaround platform for developing vaccines. Over the past decade, various recombineering methodologies have been invented that enable precise engineering of large DNA constructs on both the small and large scales. However, even following confirmation of the accuracy of the intended manipulations, off-target mutations remain a concern, especially when, as in this case, multiple iterative changes are made within a single BAC lineage. Added to this is the serious potential for mutation during reconstitution of virus from a BAC. To allay these concerns, we screened BACs initially by RFLP analysis and then assessed their full integrity by complete genome sequencing. We also sequenced various viruses reconstituted from the BACs generated in the final two steps (RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{−} BAC and RhCMV\textsubscript{68−1}/EBOV/RL11G\textsuperscript{+} BAC). Since the case was complex and the pipeline was untested, RFLP screening and genome sequencing were used extensively, often with multiple clones at each step. In total, 974 BACs were subjected to RFLP analysis and 83 BACs and 16 reconstituted viruses were examined by whole genome sequencing. Although most of the repairs were achieved as intended, some were problematic (e.g., the initial attempt at repairing RL11B at Step 7) or because off-site substitutions were introduced. In addition, sequencing the reconstituted viruses provided critical
information on genetic stability, leading to the conclusion that virus reconstituted from RhCMV\texttextsubscript{68−1}/EBOV/RL11G\texttextsuperscript{+}, unlike that reconstituted from RhCMV\texttextsubscript{68−1}/EBOV/RL11G\texttextsuperscript{−} BAC, was stable in epithelial (hTERT RPE-1) cells.

We chose to establish the HHi-FiVe pipeline by repairing RhCMV\texttextsubscript{68−1}/EBOV because of the potential of the reconstituted virus to protect rhesus macaques challenged with lethal EBOV infection by transmitted, rather than parenteral subcutaneously administered vaccination. This decision also had the advantage of involving a case in which small- and large-scale repairs were accomplished in several steps, each consisting of multiple manipulations. The success of the pipeline in this situation indicates that it is likely to be broadly applicable. The complexity of this case is inherent in the development of research on RhCMV\texttextsubscript{68−1}, which, because the virus is highly mutated, lacks key phenotypic properties, including the ability to infect non-fibroblast cells, and in the fact that all available BACs are derived from this strain. This has led to previous attempts to restore wild type properties by repairing RhCMV\texttextsubscript{68−1} BAC. The resulting BACs include one in which UL36 and the region containing UL128, UL130 and UL131A were repaired\textsuperscript{32}, and one containing a full-length genome that has been repaired more extensively\textsuperscript{23} but retains the frameshifts in RL11B, RL11D and RL11E. These repaired BACs have formed an important prelude to experimentation on the immunobiology and pathology of RhCMV in its natural host. Our repair of RhCMV\texttextsubscript{68−1}/EBOV was, by contrast, vaccine-oriented and corrected all inactivated (i.e., prematurely terminated, frameshifted, deleted, or rearranged) ORFs.

In each of the instances described above, repairs were made by identifying mutated and nonmutated sequences from genome alignments of RhCMV\texttextsubscript{68−1} and other RhCMV strains. Since all the strains had been isolated in cell culture and were themselves potentially mutated, this involved a degree of interpretation, making it difficult to be sure that all mutations had been identified. A more straightforward approach would be to construct a BAC from a strain that has been sequenced directly from the host and passaged minimally, and then to repair the BAC accordingly, as has been done with HCMV\textsuperscript{31}. However, this approach carries the inherent risk that any new BAC may represent a virus with phenotypic differences from those of RhCMV\texttextsubscript{68−1}, the immunology of which has been characterized extensively during its development as a vaccine platform (further details are below). The RhCMV case also raises the further complexity that the virus reconstituted from a repaired BAC (RhCMV\texttextsubscript{68−1}/EBOV/RL11G\texttextsuperscript{+}) is unstable, with mutants in RL11G quickly being selected in cell culture. Alternative solutions are to use a stable virus in which the problematic gene remains inactivated (RhCMV\texttextsubscript{68−1}/EBOV/RL11G\texttextsuperscript{−}) or is placed under conditional control, as has been done for both RhCMV\textsuperscript{23} and HCMV\textsuperscript{31,34}. The former solution is technically the simpler, but requires the phenotype of interest not to depend on the inactivated gene. It may be practical if the mutation is simple and can revert easily under selective pressure.

Herpesvirus-based vectors are showing considerable promise for use as conventional vaccines to control multiple pathogens that heretofore have been difficult to control\textsuperscript{3,8}. In this context, herpesvirus-based vectors, in particular those based on CMVs, have been shown to have a distinct immunological profile associated with unique T-cell based antigen recognition based on MHC-E\textsuperscript{35}. CMV-based vaccines have also been shown to provide immunological protection when administered via direct parenteral inoculation against lethal EBOV challenge in rhesus macaques, thus providing the basis for the present study\textsuperscript{5}. However, most highly pathogenic emerging viruses spill over into human populations from inaccessible wild animal populations\textsuperscript{36}, which poses a considerable limitation on the use of directly administered vaccines. The features of CMVs thus motivate the development of CMV-based vectors as a transmissible vaccine platform to achieve high immune coverage in such situations\textsuperscript{6,13,14}. Our study has provided the means and experience whereby the efficient production of precision-made, genetically validated herpesvirus-based vectors can contribute to the further development of this platform. Towards this goal, the virus reconstituted from RhCMV\texttextsubscript{68−1}/EBOV/RL11G\texttextsuperscript{−} BAC using the HHi-FiVe pipeline is currently being tested for its ability to act as a transmissible vaccine against lethal EBOV challenge in the rhesus macaque model.

**Methods**

**Cell lines**

Human telomerase-immortalized human retinal pigmented epithelial (hTERT RPE-1) cells (ATCC CRL-4000) and human telomerase-immortalized rhesus fibroblast cells (Telo-RF) cells\textsuperscript{37} were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % (v/v) fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere of 5 % (v/v) CO\textsubscript{2}. 
RhCMV$_{68-1}$-based BACs

The parental BACs used were the original RhCMV$_{68-1}$ BAC$^{17}$ and derivative (RhCMV$_{68-1}$/EBOV BAC$^{5,17}$) bearing EBOV-GP in place of RhCMV UL83B (Rh112). The circular sequence of RhCMV$_{68-1}$/EBOV BAC consists of the unique region (U) of the viral genome and three tandem copies of a direct repeat that forms the terminal direct repeat (TR) of the viral genome (one copy at each end), with the BAC vector inserted into a location in U between genes US1 (Rh181) and US2 (Rh182). The transgene consists of a synthetic codon-optimised version of the Zaire ebolavirus/H.sapiens-tc/COD/1976/Yambuku-Mayinga EBOV-GP ORF encoding the EBOV glycoprotein (GenBank accession no. AF086833.2) with its 3’ end extended to encode a 14 amino acid residue V5 epitope tag, followed by downstream noncoding sequences. Two unintended but inconsequential differences were noted in the RhCMV$_{68-1}$/EBOV clone used for repair$^5$: a non-synonymous substitution in EBOV-GP that results in an A to T amino acid substitution at codon 474 (a T residue is encoded at this position in some EBOV strains), and a noncoding substitution in the viral sequence very close to the right end of the transgene.

RhCMV$_{68-1}$/EBOV-based BAC recombineering

Mutations in RhCMV$_{68-1}$/EBOV-based BACs in *Escherichia coli* GS1783 were repaired by using lambda Red recombination and *en passant* mutagenesis as described previously$^{38}$. For small-scale repairs, a PCR product containing the repaired sequence and a selectable marker with an adjacent I-SceI restriction site was recombined into the BAC. The selectable marker was removed by the *E. coli* GS1783-encoded I-SceI endonuclease, leaving a scarless repair. RL11G was repaired by replacing the mutated ORF with a selectable marker for kanamycin resistance and then replacing this marker by a synthetic, repaired version of RL11G by using a selectable marker for spectinomycin resistance. For large-scale repairs, a 7.3 kbp region (UL/b’) [UL124 (Rh156.2) to UL145 (Rh162)] was removed using a Kan marker and then repaired in three stages by *en passant* mutagenesis using three synthetic sequences comprising a wild type version of this region based on strain 19936 (KX689268.1).

RFLP analysis

Correct lambda Red recombination and *en passant* mutagenesis were confirmed using RFLP. Mutated and repaired RhCMV$_{68-1}$/EBOV-based BACs in *E. coli* GS1783 were grown overnight in Luria-Bertani (LB) broth (Thermo Fisher Scientific) containing 17.5 µg/ml chloramphenicol. BAC DNA was extracted, digested with various restriction endonucleases, and subjected to agarose gel electrophoresis. The gels were stained with 0.5 µg/ml ethidium bromide and photographed under ultraviolet illumination to identify differences in DNA fragment mobility.

RhCMV$_{68-1}$-based BAC virus reconstitution

BAC DNA (4 µg) was transfected into resuspended cells (1x10$^6$ hTERT RPE-1 or Telo-RF cells for each well of a 6-well plate) using Genjet (SignaGen Laboratories) according to the manufacturer’s instructions. The monolayers were expanded when confluency was reached and all cells showed cytopathic effect. At this time, the cell supernatant was collected, centrifuged at 5000 x g for 10min, aliquoted and store at -80°C. DNA was extracted for complete genome analysis from an aliquot of the infected cell supernatant or a fraction of the cell lysate using an innuPREP DNA Mini kit (Analytik Jena) according to the manufacturer’s instructions.

Genome sequencing

Genome sequences were determined by standard techniques. Briefly, DNA (100 ng) from a BAC or a virus in infected cells or infected cell supernatant was sheared in a Covaris S220 sonicator to approximately 450 bp, and a sequencing library was prepared by carrying out seven cycles of PCR with indexed primers (New England Biolabs) using a Kapa LTP library preparation kit (Kapa Biosystems). Libraries were sequenced using MiSeq or NextSeq instruments (Illumina), generating datasets of 611,268–22,429,774 (BAC samples) or 2,767,034–9,488,118 (virus samples) paired-end 150 or 300 nucleotide (nt) reads per sample.

Low-quality reads and sequencing adapters were removed from the datasets using Trim Galore v. 0.4.0 (https://github.com/FelixKrueger/TrimGalore), and the remaining reads were aligned with an appropriate reference genome using Bowtie 2 v. 2.3.1$^{39}$. Alignments were visualized using Tablet v. 1.19.09.03$^{40}$. If necessary, the reference genome was corrected iteratively and fresh alignments were made. The average coverage depth of the final sequences was 136–11,175 (BAC samples) or 19–2,364 (virus samples) reads/nt. Of the BACs and viruses analysed, all but one were derived from RhCMV$_{68-1}$. The exception was sequenced to supplement Table 1, and consisted of a minor population of full-length genomes present in a stock of RhCMV strain...
180.92, which consisted mainly of genomes bearing a large deletion in UL/b\(^4\). In this case, DNA was isolated from virus generated by transfecting a historical stock of purified virion DNA into Telo-RF cells.

**Cellular tropism analysis**

Multistep growth curves were conducted in triplicate in hTERT RPE-1 cells and Telo-RF cells in 6-well plates (5x10\(^4\) cells/well) and infected at the indicated multiplicity of infection (MOI) based on plaque-forming units (PFU)/cell. At 4 h post infection (p.i.), the cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS), and fresh medium was added. Total supernatant was then collected at various times p.i., followed by washing the monolayers once with DPBS and adding fresh medium. Viral titer in the supernatant was determined by standard plaque assay on Telo-RF cells. Growth curves were performed at least twice.

**Viral transcription analysis**

Confluent hTERT RPE-1 cells in two 175 cm\(^2\) flasks were infected with RhCMV\(_{68^{-1}}/EBOV/RL11G^{-}\) at a MOI of 0.05 PFU/cell. The medium was replaced with fresh medium at 14 d p.i. At 21 d p.i., the medium was removed, and the infected cells were washed with DPBS and trypsinized using 0.05 % (w/v) trypsin in 0.02 % (w/v) ethylenediaminetetraacetic acid (EDTA) in Hank’s balanced salt solution (HBSS). The detached cells from each flask were transferred to a RNase-free 15 ml conical tube and pelleted by centrifugation at 300 x g for 5 min. The supernatant was removed, and the pellets were stored at -80\(^\circ\) C. RNA was isolated from the pellets using a RNeasy mini kit (QIAGEN), employing additional steps for virus inactivation. These included incubating at 20 \(^\circ\) C for 10 min after disrupting the cells by adding buffer RLT (QIAGEN) and incubating at 20 \(^\circ\) C for 20 min after adding 70 % (v/v) ethanol to the homogenised lysate. RNA was stored at -80 \(^\circ\) C.

Three separate DNA sequencing libraries were prepared from polyadenylated RNA selected from each sample using a TruSeq stranded mRNA library prep kit (Illumina) with IDT for Illumina TruSeq RNA UD indexes (Illumina). An aliquot of 500 ng of RNA was used for each library, and the TruSeq stranded mRNA protocol was followed with the exception that 12 PCR cycles were performed. The six indexed libraries were pooled and sequenced on a NextSeq 500/550 mid output kit v2.5 (300 cycles) (Illumina), generating approximately 40 million paired-end 150 nt reads per dataset.

Each dataset was quality-filtered using Trim Galore, sorted into sense and antisense reads using Samtools v. 1.13 (http://www.htslib.org) and mapped to the individual RhCMV\(_{68^{-1}}/EBOV/RL11G^{-}\) ORFs using Bowtie 2 with the ‘local’ option. These ORFs included that of mutated RL11G and were supplemented by the sequence encoding one long noncoding RNA (RNA4.9). The number of reads mapping to each coding region was determined by visualising the alignment using Tablet and expressed as the number of reads per kbp per million sense or antisense reads mapping to all coding regions. The relative proportion of each RNA relative to the total was then calculated as a percentage for each dataset and expressed as an average.

**EBOV-GP protein expression analysis**

Telo-RF cell monolayers in 6-well plates (5x10\(^4\) cells/well) were infected with RhCMV\(_{68^{-1}}\) (reconstituted from RhCMV\(_{68^{-1}}\) BAC) or RhCMV\(_{68^{-1}}/EBOV/RL11G^{-}\) at an MOI of 0.2 PFU/cell, and hTERT RPE-1 cells were infected with RhCMV\(_{68^{-1}}/EBOV/RL11G^{-}\) at an MOI of 0.4 PFU/cell. At various times p.i., the medium was removed, and the monolayers were lysed in boiling 2 x SDS-PAGE sample buffer (125 mM Tris-HCl pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol and 10% (v/v) 2-mercaptoethanol). Equal volumes of cell lysates were subjected to SDS-PAGE followed by semi-dry transfer to nitrocellulose membranes (GE Healthcare). Primary antibodies were applied at the dilutions indicated: anti-EBOV-GP protein (mAb clone 12/1.1; courtesy of Dr Ayato Takada; 1:10,000), anti-RhCMV UL44 protein (courtesy of Dr Thomas Shenk; 1:2) and anti-GAPDH (mAb clone 14C10, Cell Signalling; 1:1000). Compared to antibodies directed against the V5 epitope tag incorporated into EBOV GP expressed by the RhCMV\(_{68^{-1}}/EBOV\) vectors, the anti-EBOV-GP mAb gave consistently higher signal and was used for expression analysis. Secondary antibodies (horseradish peroxidase (HRP)-labeled; Dako) were applied at the dilutions indicated: anti-mouse HRP (1:5,000) and anti-rabbit HRP (1:5,000). The membranes were incubated overnight with primary antibody at 4\(^\circ\) C, and then for 1 h at room temperature with the secondary antibody in TBS-T (0.1% (v/v) Tween-20, 50 mM Tris and 150 mM NaCl, pH 7.5) containing 5 % (w/v) skimmed milk, and then washed three times for 5 min with TBS-T. The target proteins on the membranes were visualised by enhanced chemiluminescence (GE Healthcare) and imaged using a Fusion Capture Advance FX7 16.15 (Peqlab) instrument.

**Declarations**
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AUTHOR CONTRIBUTIONS

M.A.J, A.J.D., W.B. and P.A.B. conceived and designed the study with assistance from members of the UCD PREEMPT Consortium. M.A.J., A.J.D., W.B., and P.A.B., drafted the manuscript with assistance from E.O., T.M., J.N., V.M. and K.A.S. The experiments were performed by T.M., E.O., J.N., Y.W., S.H., A.K., H.N., and R.A. A.J.D. carried out sequence analysis with assistance from J.N., M.V., and J.H. All authors reviewed the manuscript.

DATA AVAILABILITY

The data supporting the findings of this study are available from public databases as stated or from the corresponding author upon reasonable request.

COMPETING INTERESTS

M.A.J., T.M., Y.W., S.H., and H.N are employed, at least in part, by TVG. The remaining authors declare no competing interests.

References

1. Ampe, B., et al. Assessment of the long-term effect of vaccination on transmission of infectious bovine rhinotracheitis virus in cattle herds hyperimmunized with glycoprotein E-deleted marker vaccine. Am J Vet Res 73, 1787–1793 (2012).
2. Parker, D., de Wit, S., Houghton, H. & Prandini, F. Assessment of impact of a novel infectious bursal disease (IBD) vaccination programme in breeders on IBD humoral antibody levels through the laying period. Vet Rec Open 1, e000016 (2014).
3. Hansen, S.G., et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature 473, 523–527 (2011).
4. Hansen, S.G., et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat Med 15, 293–299 (2009).
5. Marzi, A., et al. Cytomegalovirus-based vaccine expressing Ebola virus glycoprotein protects nonhuman primates from Ebola virus infection. Sci Rep 6, 21674 (2016).
6. Tsuda, Y., et al. A replicating cytomegalovirus-based vaccine encoding a single Ebola virus nucleoprotein CTL epitope confers protection against Ebola virus. PLoS Negl Trop Dis 5, e1275 (2011).
7. Tsuda, Y., et al. A cytomegalovirus-based vaccine provides long-lasting protection against lethal Ebola virus challenge after a single dose. Vaccine 33, 2261–2266 (2015).
8. Hansen, S.G., et al. Prevention of tuberculosis in rhesus macaques by a cytomegalovirus-based vaccine. Nat Med 24, 130–143 (2018).
9. Beverley, P.C., et al. A novel murine cytomegalovirus vaccine vector protects against Mycobacterium tuberculosis. J Immunol 193, 2306–2316 (2014).
10. Hansen, S.G., et al. Cytomegalovirus vectors expressing Plasmodium knowlesi antigens induce immune responses that delay parasitemia upon sporozoite challenge. PLoS One 14, e0210252 (2019).
11. Jarvis, M.A., Hansen, S.G., Nelson, J.A. & Fruh, K. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. Vol. 2 (ed. Reddehase, M.J.) (Caister Academic Press, Norwich, UK., 2013).
12. Hansen, S.G., et al. Evasion of CD8+ T cells is critical for superinfection by cytomegalovirus. Science 328, 102–106 (2010).
13. Murphy, A.A., Redwood, A.J. & Jarvis, M.A. Self-disseminating vaccines for emerging infectious diseases. Expert Rev Vaccines 15, 31–39 (2016).
14. Nuismer, S.L., et al. Bayesian estimation of Lassa virus epidemiological parameters: Implications for spillover prevention using wildlife vaccination. *PLoS Negl Trop Dis* **14**, e0007920 (2020).

15. Ruzsics, Z., Borst, E.M., Brune, W. & Messerle, M. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. Vol. 1 (ed. Reddehase, M.J.) 38-58 (Caister Academic Press, Norwich, UK., 2013).

16. Asher, D.M., Gibbs, C.J., Jr., Lang, D.J., Gajdusek, D.C. & Chanock, R.M. Persistent shedding of cytomegalovirus in the urine of healthy Rhesus monkeys. *Proc Soc Exp Biol Med* **145**, 794–801 (1974).

17. Chang, W.L. & Barry, P.A. Cloning of the full-length rhesus cytomegalovirus genome as an infectious and self-excisable bacterial artificial chromosome for analysis of viral pathogenesis. *J Virol* **77**, 5073–5083 (2003).

18. Rivailler, P., Kaur, A., Johnson, R.P. & Wang, F. Genomic sequence of rhesus cytomegalovirus 180.92: insights into the coding potential of rhesus cytomegalovirus. *J Virol* **80**, 4179–4182 (2006).

19. Oxford, K.L., et al. Protein coding content of the ULb' region of wild-type rhesus cytomegalovirus. *Virology* **373**, 181–188 (2008).

20. Malouli, D., et al. Reevaluation of the coding potential and proteomic analysis of the BAC-derived rhesus cytomegalovirus strain 68-1. *J Virol* **86**, 8959–8973 (2012).

21. Burwitz, B.J., et al. Cross-Species Rhesus Cytomegalovirus Infection of Cynomolgus Macaques. *PLoS Pathog* **12**, e1006014 (2016).

22. Davison, A.J., et al. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. Vol. 1 (ed. Reddehase, M.J.) (Caister Academic Press, Norwich, UK., 2013).

23. Taher, H., et al. In vitro and in vivo characterization of a recombinant rhesus cytomegalovirus containing a complete genome. *PLoS Pathog* **16**, e1008666 (2020).

24. Hansen, S.G., Strelow, L.I., Franchi, D.C., Anders, D.G. & Wong, S.W. Complete sequence and genomic analysis of rhesus cytomegalovirus. *J Virol* **77**, 6620–6636 (2003).

25. Gill, R.B., et al. Coding potential of UL/b' from the initial source of rhesus cytomegalovirus Strain 68-1. *Virology* **447**, 208–212 (2013).

26. Oxford, K.L., et al. Open reading frames carried on UL/b' are implicated in shedding and horizontal transmission of rhesus cytomegalovirus in rhesus monkeys. *J Virol* **85**, 5105–5114 (2011).

27. Wagner, M., Ruzsics, Z. & Koszinowski, U.H. Herpesvirus genetics has come of age. *Trends Microbiol* **10**, 318–324 (2002).

28. Tischer, B.K., Smith, G.A. & Osterrieder, N. En passant mutagenesis: a two step markerless red recombination system. *Methods Mol Biol* **634**, 421–430 (2010).

29. Britt, W.J., Jarvis, M., Seo, J.Y., Drummond, D. & Nelson, J. Rapid genetic engineering of human cytomegalovirus by using a lambda phage linear recombination system: demonstration that pp28 (UL99) is essential for production of infectious virus. *J Virol* **78**, 539–543 (2004).

30. Dargan, D.J., et al. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. *J Gen Virol* **91**, 1535–1546 (2010).

31. Stanton, R.J., et al. Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication. *J Clin Invest* **120**, 3191–3208 (2010).

32. Lilja, A.E. & Shenk, T. Efficient replication of rhesus cytomegalovirus variants in multiple rhesus and human cell types. *Proc Natl Acad Sci U S A* **105**, 19950–19955 (2008).

33. Wang, D. & Shenk, T. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. *Proc Natl Acad Sci U S A* **102**, 18153–18158 (2005).

34. Murrell, I., et al. Genetic Stability of Bacterial Artifical Chromosome-Derived Human Cytomegalovirus during Culture In Vitro. *J Virol* **90**, 3929–3943 (2016).

35. Hansen, S.G., et al. Broadly targeted CD8(+) T cell responses restricted by major histocompatibility complex E. *Science* **351**, 714–720 (2016).

36. Jones, K.E., et al. Global trends in emerging infectious diseases. *Nature* **451**, 990–993 (2008).

37. Chang, W.L., Kirchoff, V., Pari, G.S. & Barry, P.A. Replication of rhesus cytomegalovirus in life-expanded rhesus fibroblasts expressing human telomerase. *J Virol Methods* **104**, 135–146 (2002).
38. Tischer, B.K., von Einem, J., Kaufer, B. & Osterrieder, N. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. *Biotechniques* **40**, 191–197 (2006).

39. Langmead, B. & Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**, 357–359 (2012).

40. Milne, I., et al. Using Tablet for visual exploration of second-generation sequencing data. *Brief Bioinform* **14**, 193–202 (2013).

41. Assaf, B.T., et al. Limited dissemination and shedding of the UL128 complex-intact, UL/b'-defective rhesus cytomegalovirus strain 180.92. *J Virol* **88**, 9310–9320 (2014).

42. Marshall, E.E., et al. Enhancing safety of cytomegalovirus-based vaccine vectors by engaging host intrinsic immunity. *Sci Transl Med* **11**(2019).

**Supplementary Information**

**Supplementary Figure 1** is available in the Supplemental Files section.

**Supplementary Figure 1. Example of RFLP analysis of repaired RhCMV<sub>68-1</sub>/EBOV-based BACs.**

RFLP analysis of the RhCMV<sub>68-1</sub>/EBOV-based BAC in which RL11B was repaired (Step 7 in Table 2). The white dots mark fragments that are present in the parental BAC or the repaired BACs (results of three clones are shown).

**Supplementary Table 1. Relative abundance of sense transcripts from RhCMV<sub>68-1</sub>/EBOV/RL11G<sup>+</sup> coding regions.**
| Genea    | Proteinb                                      | % transcripts |
|----------|-----------------------------------------------|---------------|
| UL146D   | chemokine vCXCL7                             | 14.99         |
| UL22A    | glycoprotein UL22A                            | 11.91         |
| UL146C   | chemokine vCXCL6                             | 7.40          |
| RNA4.9   | [long noncoding RNA]                         | 4.41          |
| UL132    | envelope glycoprotein UL132                   | 3.43          |
| EBOV-GP  | EBOV-GP                                      | 2.31          |
| UL84     | protein UL84                                 | 2.21          |
| UL40     | membrane glycoprotein UL40                   | 2.00          |
| UL30A    | protein UL30A                                | 1.96          |
| UL99     | myristylated tegument protein                 | 1.59          |
| UL71     | tegument protein UL51                        | 1.47          |
| UL82     | tegument protein pp71                        | 1.06          |
| UL80     | capsid maturation protease and scaffold protein | 1.05          |
| UL148    | membrane protein UL148                       | 1.03          |
| UL42     | protein UL42                                 | 0.84          |
| UL48A    | small capsid protein                          | 0.83          |
| UL34     | protein UL34                                 | 0.83          |
| UL17     | protein UL17                                 | 0.83          |
| UL26     | tegument protein UL26                        | 0.81          |
| UL85     | capsid triplex subunit 2                     | 0.74          |
| UL115    | envelope glycoprotein L                       | 0.71          |
| UL43     | tegument protein UL43                        | 0.70          |
| UL122 (exon 3) | regulatory protein IE2              | 0.69          |
| UL128    | envelope protein UL128                       | 0.67          |
| UL92     | protein UL92                                 | 0.67          |
| O25      | protein O25                                  | 0.66          |
| UL94     | tegument protein UL16                        | 0.64          |
| UL112    | protein UL112                                | 0.63          |
| UL87     | protein UL87                                 | 0.62          |
| US12     | membrane protein US12                        | 0.59          |
| UL32     | tegument protein pp150                       | 0.59          |
| UL41A    | protein UL41A                                | 0.57          |
| UL88     | tegument protein UL88                        | 0.56          |
| RL111    | membrane protein RL111                       | 0.56          |
| UL98     | deoxyribonuclease                            | 0.54          |
| UL91     | protein UL91                                 | 0.53          |
| UL45     | ribonucleotide reductase subunit 1           | 0.52          |
| UL78     | envelope protein UL78                        | 0.50          |
| UL49     | protein UL49                                 | 0.50          |
| UL74A    | envelope glycoprotein 24                     | 0.45          |
| UL144    | membrane glycoprotein UL144                  | 0.43          |
| UL111A   | interleukin-10                               | 0.43          |
| UL46     | capsid triplex subunit 1                     | 0.41          |
| UL100    | envelope glycoprotein M                      | 0.40          |
| US30     | membrane protein US30                        | 0.39          |
| US6C     | protein US6C                                 | 0.38          |
| UL14     | membrane protein UL14                        | 0.38          |
| UL130    | envelope glycoprotein UL130                  | 0.36          |
| UL44     | DNA polymerase processivity subunit           | 0.36          |
| UL75     | envelope glycoprotein H                      | 0.36          |
| O12      | protein O12                                 | 0.35          |
| UL147    | chemokine vCXCL2                             | 0.33          |
| US6      | membrane glycoprotein US6                    | 0.33          |
| COX2     | prostaglandin G/H synthase 2                 | 0.32          |
| UL73     | envelope glycoprotein N                      | 0.32          |
| UL38     | protein UL38                                 | 0.32          |
| US22     | tegument protein US22                        | 0.32          |
| UL146B   | chemokine vCXCL5                             | 0.32          |
| UL103    | tegument protein UL7                         | 0.31          |
| UL116    | protein UL116                                | 0.30          |
| UL147A   | membrane protein UL147A                      | 0.27          |
| US27A    | membrane protein US27A                       | 0.27          |
| UL53     | nuclear egress lamina protein                | 0.27          |
| US27E    | membrane protein US27E                       | 0.26          |
| UL83A    | protein UL83A                                | 0.25          |
| UL123 (exon 3) | regulatory protein IE1              | 0.25          |
| UL146F   | chemokine vCXCL9                             | 0.25          |
| O19      | protein O19                                 | 0.24          |
| UL31     | protein UL31                                | 0.24          |
| US17     | membrane protein US17                        | 0.23          |
| O13      | protein O13                                 | 0.22          |
| Protein/ID | Description | Score |
|-----------|-------------|-------|
| O24       | Protein O24 | 0.22  |
| RL11J     | Membrane protein RL11J | 0.21  |
| UL55      | Envelope glycoprotein B | 0.21  |
| UL146H    | Chemokine vCXCL11 | 0.21  |
| UL114     | Uracil-DNA glycosylase | 0.20  |
| UL145     | Protein UL145 | 0.20  |
| UL30      | Protein UL30 | 0.20  |
| O21       | Protein O21 | 0.20  |
| UL86      | Major capsid protein | 0.19  |
| UL52      | DNA packaging protein UL32 | 0.19  |
| RS1       | Tegument protein TRS1 | 0.19  |
| US18      | Membrane protein US18 | 0.18  |
| UL20      | Membrane protein UL20 | 0.18  |
| UL33      | Envelope glycoprotein UL33 | 0.18  |
| UL131A    | Envelope protein UL131A | 0.18  |
| UL54      | DNA polymerase catalytic subunit | 0.18  |
| UL25      | Tegument protein UL25 | 0.17  |
| UL36      | Tegument protein vCA | 0.17  |
| US27B     | Membrane protein US27B | 0.17  |
| RL1       | Protein RL1 | 0.17  |
| O7        | Protein O7 | 0.17  |
| UL35      | Tegument protein UL35 | 0.17  |
| UL141     | Membrane glycoprotein UL141 | 0.16  |
| RL11H     | Membrane protein RL11H | 0.16  |
| UL93      | DNA packaging tegument protein UL17 | 0.16  |
| UL21A     | Protein UL21A | 0.15  |
| O9        | Protein O9 | 0.15  |
| RL11A     | Membrane protein RL11A | 0.15  |
| US29      | Membrane protein US29 | 0.15  |
| O23       | Protein O23 | 0.14  |
| US32      | Protein US32 | 0.14  |
| UL50      | Nuclear egress membrane protein | 0.13  |
| UL69      | Multifunctional expression regulator | 0.13  |
| UL13      | Protein UL13 | 0.13  |
| US12E     | Membrane protein US12E | 0.13  |
| US12      | Membrane protein US12A | 0.13  |
| UL117     | Protein UL117 | 0.12  |
| UL57      | Single-stranded DNA-binding protein | 0.12  |
| UL37      | Envelope glycoprotein UL37 | 0.11  |
| O14       | Protein O14 | 0.11  |
| UL23      | Tegument protein UL23 | 0.11  |
| UL72      | Deoxuryridine triphosphatase | 0.11  |
| US3       | Membrane glycoprotein US3 | 0.11  |
| US26      | Protein US26 | 0.10  |
| UL119     | Membrane glycoprotein UL119 | 0.10  |
| US27D     | Membrane protein US27D | 0.10  |
| UL89      | DNA packaging terminase subunit 1 | 0.10  |
| US28      | Envelope protein US28 | 0.10  |
| UL77      | DNA packaging tegument protein UL25 | 0.10  |
| RL11T     | Membrane protein RL11T | 0.09  |
| UL104     | Capsid portal protein | 0.09  |
| US11      | Membrane glycoprotein US11 | 0.08  |
| UL27      | Protein UL27 | 0.08  |
| UL105     | Helicase-primase helicase subunit | 0.08  |
| US31      | Protein US31 | 0.07  |
| US20      | Membrane protein US20 | 0.07  |
| US6A      | Membrane glycoprotein US6A | 0.07  |
| UL97      | Tegument serine/threonine protein kinase | 0.06  |
| US1       | Protein US1 | 0.06  |
| UL124     | Membrane protein UL124 | 0.05  |
| UL19      | Protein UL19 | 0.05  |
| O15       | Protein O15 | 0.05  |
| UL56      | DNA packaging terminase subunit 2 | 0.05  |
| US19      | Membrane protein US19 | 0.05  |
| RL11G     | Membrane protein RL11G | 0.05  |
| O22       | Protein O22 | 0.05  |
| UL79      | Protein UL79 | 0.05  |
| O10       | Protein O10 | 0.05  |
| UL51      | DNA packaging protein UL33 | 0.05  |
| US2       | Membrane glycoprotein US2 | 0.04  |
| UL48      | Large tegument protein | 0.04  |
| US6B      | Membrane glycoprotein US6B | 0.04  |
From Fig. 1 and GenBank accession no. MZ517253.1.

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Number of sense transcripts from individual coding regions as a percentage of the number of sense and antisense transcripts from all coding regions. Ranked from the greatest number of transcripts to the least. Accuracy is limited by several factors. First, values for small coding regions may be depressed because of reduced mapping of reads to their ends. For example, this may lead to an underestimate of the levels of expression of UL146D and UL22A (first and second, respectively, in the list). Second, expression of upstream coding regions in a family of overlapping mRNAs sharing a 3’-end will enhance the apparent level of expression of downstream coding regions. For example, this may lead to an overestimate of the level of expression of UL146D (first in the list), which is probably 3’-coterminal with UL146C (third in the list); this would make UL22A first in the list. Third, a proportion of antisense transcripts may have been generated from overlapping noncoding regions of sense transcripts from adjacent genes. For example, UL89 (exon 1) is overlapped by the 3’-end of the more highly expressed UL94 on the opposite strand. This factor may also lead to an overestimate of the overall proportion of antisense transcripts (6.84%).

Supplementary Table 2. Primers used for repair of ORFs in RhCMV68-1/EBOV
| Name | Sequence | Purpose |
|------|----------|---------|
| Rh157.4 | GTACGATACTGTACGGTTGTGATTTTGAAGTCATAGCAGTCCTGTG TAGGGATAACAGGGTAATCGATTT | Introduction of I-SceI sequences and a kanamycin resistance gene in the shuttle plasmid containing UL124 to UL132 |
| Rh157.4 | GTAGCCAGTGTACACCAATTAACC | |
| Rh157.4 Rh157.5 Rh157.6 | AGCCGTCTACATACGGACACCACCCTTTAAGTGTCTTATACAGTGATTTATGTGCTTTAACAATACCGGCAAGTAAGCCTG | Amplification of fragment containing UL130 to UL131A and introduction of a kanamycin resistance gene |
| Rh157.5 | TCCCTACTATAATAACGTGCTG | |
| Rh157.5 Reverse | AGCCGTCTACATACGGACAC | Amplification of fragment containing UL128 and introduction of a kanamycin resistance gene |
| Rh157.5 | TGTTAGGCGGCACTCCTTC | |
| Rh13.1 | AAACAGTGCAATACGATACAGTTGCTTCATCGATGTACGGCGGTAGGATAACAGGTAATCGATTT | Removal of RL11G and introduction of a kanamycin resistance gene |
| Rh13.1 | AAACAGTGCAATACGATACAGTTGCTTCATCGATGTACGGCGGTAGGATAACAGGTAATCGATTT | |

**Figures**
Figure 1

Genetic map of RhCMV68-1/EBOV/RL11G+ BAC. The circular sequence is depicted in linear form, starting at the left end of the viral genome with one copy of the terminal direct repeat (TR), proceeding through the unique region (U), and ending with two more copies of TR. The copies of TR are shown in a thicker format than U. Protein-coding ORFs are indicated by coloured open arrows grouped according to the key at the foot to indicate gene families, other non-core genes that are not conserved among herpesviruses and core genes that are conserved among herpesviruses. Introns connecting protein-coding regions are shown as narrow white bars. UL72 is both a core gene and member of the DURP gene family and is shown as the latter. The BAC vector is shown by the grey-shaded region between US1 and US2. The EBOV-GP ORF encoding a V5-tagged EBOV spike glycoprotein is also grey-shaded and replaced US83B. The locations of small-scale and large-scale repairs (see Table 2) are marked above the genome by yellow squares and bars, respectively.
Figure 2

Schematic showing the HHi-FiVe pipeline. i) Lambda Red recombination and en passant mutagenesis are used to repair individual ORFs in the BAC, ii) the repaired BAC is screened by using RFLP, and then iii) Illumina sequencing, iv) read mapping v) accompanied by analysis are used to confirm the intended repair and the absence of off-site mutations. The process is repeated until all repairs are made.
Reconstitution and passaging of viruses for sequencing. Clones of RhCMV68-1/EBOV/RL11G– BAC and RhCMV68-1/EBOV/RL11G+ BAC (denoted RL11G– BAC and RL11G+ BAC, respectively; shaded grey) were transfected into Telo-RF (F; shaded blue) or hTERT RPE-1 (E; shaded green) cells or a 1:1 mixture of these cells (E+F; shaded orange). The transfected cells were passaged further (p, passage number), and, in some instances, virus stocks (Ⅲ) were made. The hatched triangle indicates that a stock was made from a mixture of yields from several passages. Hatched arrows indicate recombineering, light grey arrows indicate transfection, and dark grey arrows indicate passaging and expansion. Samples A–P (red font) were sequenced from DNA isolated from infected cells (black border of the rounded rectangles) or infected cell supernatant (red border of the rounded rectangles).
Cellular tropism of RhCMV68-1/EBOV/RL11G– compared to parental RhCMV68-1/EBOV-GP. Multi-step growth analysis was conducted by infecting Telo-RF (circles) or hTERT RPE-1 (squares) cells at a MOI of 0.2 PFU/cell with the reconstituted viruses RhCMV68-1/EBOV-GP (closed symbols) or RhCMV68-1/EBOV/RL11G– (open symbols). Supernatant was collected at the indicated d p.i. and titrated using a plaque assay. Titers are shown as ± SEM. DL, detection limit.
Figure 5

EBOV-GP expression of reconstituted viruses. A) Telo-RF cells were infected at a MOI of 0.2 PFU/cell with either RhCMV68-1 or RhCMV68-1/EBOV/RL11G−. B) hTERT RPE-1 cells were infected at a MOI of 0.4 PFU/cell with RhCMV68-1/EBOV/RL11G−. In both experiments, cell lysates were collected at the indicated d.p.i and analyzed by immunoblotting. EBOV-GP was detected by using an EBOV GP-specific mAb. An antibody against the RhCMV UL44 protein was used as a viral infection control, and a mAb against cellular GAPDH was used as a cellular protein loading control.

Supplementary Files

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