Transient dominant host-range selection using Chinese hamster ovary cells to generate marker-free recombinant viral vectors from vaccinia virus

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Recombinant vaccinia viruses (rVACVs) are promising antigen-delivery systems for vaccine development that are also useful as research tools. Two common methods for selection during construction of rVACV clones are (i) co-insertion of drug resistance or reporter protein genes, which requires the use of additional selection drugs or detection methods, and (ii) dominant host-range selection. The latter uses VACV variants rendered replication-incompetent in host cell lines by the deletion of host-range genes. Replicative ability is restored by co-insertion of the host-range genes, providing for dominant selection of the recombinant viruses. Here, we describe a new method for the construction of rVACVs using the cowpox CP77 protein and unmodified VACV as the starting material. Our selection system will expand the range of tools available for positive selection of rVACV during vector construction, and it is substantially more high-fidelity than approaches based on selection for drug resistance.

Recombinant viral vectors offer a promising method for antigen delivery in new vaccine and therapeutic agent formulations, with vaccinia virus (VACV) (Fam. *Poxviridae*) being one of the most

developed viruses in vaccine research (1). The choice of VACV to generate new recombinant viral vectors is motivated by its ability to elicit strong, sustained humoral and cell-mediated responses

(2), its tolerance of large DNA insertions into its genome (3), and its lack of genomic integration in the host due to cytoplasmic replication (4).

Recombinant VACV (rVACV) variants containing novel sequences and/or deleted viral genes are routinely produced by homologous recombination (HR) events between DNA sequences contained in a shuttle vector (usually plasmid DNA) and the VACV target sequences (5). Two different methods are often used to identify and isolate new recombinant virus clones. The first relies on the insertion of drug resistance (6) and/or reporter protein genes (7); the former enables amplification of the rVACV while replication of the parent virus is suppressed by the specific drug, whereas the latter allows identification of the rVACV by its expression of the reporter protein. The second method, termed dominant host-range selection, is used to select the recombinant virus in a stringent growth selection cell line (i.e., one that does not permit VACV replication) by introducing the missing host-range gene. This method has been described previously for the construction of a recombinant modified vaccinia Ankara (MVA) in the RK-13 cell line (derived from rabbit kidney cells), which is non-permissive for MVA infection, by inserting the VACV host-range gene *K1L*. In MVA, the *K1L*-equivalent ORF is severely truncated and non-functional, but when function is restored by insertion of VACV-*K1L* into the MVA genome, this rMVA is capable of multiplying in RK-13 cells (8). However, as VACV has a functional *K1L* gene, this method is not applicable for rVACV construction. Several other methods for rVACV construction are based on prior construction of a progenitor rVACV in which the host-range gene for replication in the chosen cell line has been deleted (9,10). This progenitor rVACV is replication-incompetent; however, after the next genetic modification, competency is restored by recombination and insertion of

METHOD SUMMARY

Here, we describe the first transient dominant host-range selection method for generating recombinant vaccinia viruses (rVACVs) in Chinese hamster ovary (CHO) cells through expression of the cowpox host-range gene coding for the CP77 protein to obtain marker-free rVACVs in a VACV-permissive cell line.

the host-range gene to generate the desired rVACV clone.

Vaccinia virus has a broad host range but cannot complete its replication cycle in Chinese hamster ovary (CHO) cells (11,12). However, replication can be rescued by insertion of the *CP77* gene from the cowpox virus (13). Here, we describe a new transient dominant host-range selection approach using VACV and CHO cells to facilitate rapid construction and efficient selection of rVACV without prior construction of an rVACV progenitor. This was achieved by incorporating the *CP77* gene in the design of the shuttle vector used to generate the resulting rVACV. We demonstrated this new method by replacing the semaphorin *A39R* ORF with a fluorescent protein expression cassette in the VACV Copenhagen strain (VACV-COP), with subsequent deletion of the fluorescent protein expression cassette once removal of the *A39R* ORF was confirmed.

Chinese hamster ovary cells (ATCC CCL61) and baby hamster kidney cells (BHK-21, ATCC CCL10) were used to generate the final marker-free *A39R*-rVACV. Synthesized DNA constructs (GeneArt/Thermo Fisher Scientific, Regensburg, Germany) and standard recombinant DNA methods were used to produce the pA39R-Del recombination plasmid. Briefly, pA39R-Del consisted of two HR arm sequences (F1 and F2) matching the flanking regions of the VACV *A39R* locus. The *CP77* gene (cowpox virus strain Brighton Red CPXV025, Genbank ID M19531), with its native promoter (100 bp upstream of the start codon), and the *Discosoma* red fluorescent protein open reading frame (*DsRed*) (Genbank

Figure 1. Creation of a *DsRed*⁺ *A39R*⁻ intermediate recombinant vaccinia virus (rVACV) using host-range gene *CP77* as a selectable marker in VACV non-permissive Chinese hamster ovary (CHO) cells. (A) Recombinant irVACV-DsRed was constructed by DNA homologous recombination (HR) using a shuttle vector containing an expression cassette flanked by arms F1 and F2 matching the flanking sequences of the VACV Copenhagen strain (VACV-COP) *A39R* gene locus. The gene expression cassette insertion consisted of the *CP77* and *DsRed* genes under the control of the native *CP77* promoter and a synthetic early/late poxviral promoter (prPs), respectively. The small black arrows indicate the locations of the ID-A39R and A39R-FL primers. (B) Red (*DsRed*⁺) fluorescent irVACV-DsRed plaques, formed in CHO cells after HR between the shuttle vector and VACV-COP, at 1–3 days post-infection. (C) Following 5 rounds of plaque purification, an internal PCR primer pair designed to amplify a 801-bp region of the *A39R* gene was used to detect parental VACV-COP. No product was seen in PCR of *A39R*⁻ clones. Lane M: 2-log DNA ladder (New England Biolabs); Lanes 1–7: irVACV-DsRed clones 1–7; and Lane V: VACV-COP. (D) DNA quality was tested by PCR amplification of the VACV-COP *ATI* gene locus region.

ID FJ226078), with a synthetic vaccinia early/late promoter (14), were placed between F1 and F2. The F1 and F2 arms were each 1000-bp long in order to mediate efficient homologous recombination. Additionally, a 250-bp repeat (Rpt) of the 3' end of F1 was inserted at the 3' end of *DsRed* to allow for the excision of the expression cassette by

intramolecular recombination between F1 and F1-Rpt in the absence of CHO host-range selection (15) (Figure 1A).

To achieve HR between the shuttle vector and the VACV-COP *A39R* locus, CHO cells were infected with VACV-COP at a multiplicity of infection (moi) of 0.01. After 45 min, infected cells were transfected with 1 µg of *NotI*-HF (New England Biolabs, Ipswich, MA)-linearized pA39R-Del using Effectene (Qiagen, Doncaster, Australia). Cells were harvested 1 day later, and viral extracts were prepared by multiple freeze/thaw cycles. Recombinant VACV plaque purification was performed by serially diluting the viral extract 1:10,000 and then re-infecting CHO cells with the diluted extract. Two days post-infection, single fluorescent plaques (*DsRed*⁺), indicative of cells infected with intermediate rVACV (irVACV-DsRed) containing the *CP77*-*DsRed* expression cassette after

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Figure 2. Deletion of the *CP77-DsRed* expression cassette from the intermediate recombinant vaccinia virus (rVACV)-DsRed in the VACV permissive cell line BHK-21 by intramolecular recombination. (A) In VACV-permissive BHK-21 cells, *irVACV-DsRed* can recombine to delete the *CP77-DsRed* expression cassette by DNA homologous recombination (HR) between the 250-bp repeat (Rpt) sequences, generating marker-free *A39R⁻ rVACV*. The small black arrows indicate the locations of the A39R-FL primers. (B) Following 2–3 rounds of plaque purification, no parental VACV-COP was detected by PCR in the *A39R⁻ rVACV*. (C) Flanking-region PCR confirmed the absence of the reporter cassette in the *A39R⁻ rVACV*. The assay was designed to span the *A37R* to *A41L* region such that the *A39R⁻ rVACV* yields 2449-bp PCR products, while the parental VACV-COP and *irVACV-DsRed* yield larger PCR products (3497 bp and 5643 bp, respectively). (D) DNA quality was tested by PCR amplification of the VACV *ATI* gene locus region. Lane M: 2-log DNA ladder; Lane R: genomic *A39R⁻ rVACV* DNA; Lane I: genomic *irVACV-DsRed* DNA; Lane V: genomic VACV-COP DNA.

HR, were identified (Figure 1B) and harvested to prepare new viral extracts. After five rounds of purification, PCR analysis confirmed that the *A39R* gene

was absent in *irVACV-dsRed* clones (Figure 1C). For all PCR analyses, a housekeeping PCR assay that amplified a section of the VACV-COP

ATI locus was included to validate the quality of the DNA template (Figure 1D); the primer pairs are described in Table 1. In addition, a flanking PCR

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was performed, which confirmed the replacement of the *A39R* gene by the selection cassette in the irVACV-dsRed clone compared with the parental VACV (Figure 2C; discussed below).

In the second stage of the process, the *CP77-DsRed* expression cassette was deleted. To that end, VACV replication-permissive BHK-21 cells were infected with purified irVACV-DsRed clones. As *CP77* was not required for replication in that cell line, the generation of marker-free *A39R*⁻ rVACV (*i.e.*, containing neither the *CP77-DsRed* nor the *A39R* genes) was enabled via an intramolecular recombination event mediated by the Rpt sequences (Figure 2A). After 2–3 rounds of purification, the *A39R*⁻ rVACV was isolated and purified from individual non-fluorescent plaques (*DsRed*⁻) and analyzed by PCR to confirm the absence of both the *A39R* locus (Figure 2B) and the *CP77-DsRed* reporter cassette (Figure 2C). Notably, two PCR products were generated from the irVACV-DsRed DNA template, demonstrating

Table 1. Primer pairs for PCR analysis.

Primer	Sequence (5' to 3')	Amplicon size (bp)		
		VACV	irVACV- <i>DsRed</i>	<i>A39R</i> rVACV
ID-A39R-Fw	AGAGGATGCGGATAAGGATACATTAGTATGC	801	-	-
ID-A39R-Rv	TCCTTTATATCGAACCATTTAACACCAGACG			
A39R-FL-Fw	GTCGTACAATTCTGTACCTATCAAGG	3497	5643	2473
A39R-FL-Rv	CGCATCTGTATCAAACGGAGG			
COP-AT1-Fw	CGAGCATTACTGCGTTTAAATCTAT	513		
COP-AT1-Rv	TAAACCATAAATACATGGGTGATCC			

ID-A39R primers amplify a section of the *A39R* locus.
A39R-FL primers are located outside of the HR flanking regions, span the VACV Copenhagen strain (VACV-COP) *A37R* to *A41L* genomic region.
COP-AT1 primers amplify the *AT1* locus in VACV-COP.

that excision of the marker cassette occurred during the selection in CHO cells, which is consistent with previous observations (8). The quality of the DNA was similarly tested as described above (Figure 2D). *A39R*⁻ rVACV PCR products amplified using the A39R-FL primers were sequenced and aligned with the reference sequence to confirm deletion of the *A39R* gene and removal of the *CP77-DsRed* cassette (data not shown). In addition, *A39R*⁻ rVACV was used to infect CHO cells. No viral expansion (fluorescent or

non-fluorescent) was observed (data not shown), which further verified successful removal of the reporter cassette.

Since the pioneering work of Perkus et al. demonstrating that VACV could be genetically modified to impede or allow its replication in growth-restricted cell lines (9), many different variations of the dominant host-range selection method have been reported and have been used to generate rVACV (8,10). By taking advantage of the ability of the cowpox *CP77* protein to rescue VACV growth in the otherwise non-permissive CHO cell line (11,13), we were able to replace the *A39R* locus in VACV-COP with an expression cassette (*CP77-DsRed*) without the need for constructing a precursor host-range-restricted rVACV. Furthermore, the shuttle vector was designed to obtain marker-free rVACV, which is desirable for translational applications or to enable reuse of this selection method for further modifications to the rVACV genome. Host-range selection provides for positive selection of rVACV while inhibiting parental virus replication for efficient rVACV purification, all without the additional cost of drug selection. Our method therefore combines the advantages of conventional dominant drug resistance and color selection for the efficient and economical production of rVACV and adds a new option to the list of rVACV selection methods.

Author contributions

P.H. conceived and commissioned the project. L.L., T.C., P.E., P.H., and J.H. designed the experiments. L.L. performed the experiments and analyzed the results. L.L., T.C., P.E.,

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P.G.-V., and K.D. prepared the figures and wrote the manuscript. All authors discussed the results and edited the manuscript. P.H., K.D., and J.H. supervised the project. The principal investigator was J.H.

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Competing interests

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