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Transient Host Range Selection for Genetic Engineering of Modified Vaccinia Virus Ankara

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ABSTRACT

Recombinant vaccinia viruses are extremely valuable tools for research in molecular biology and immunology. The extension of vaccinia vector technology to replication-deficient and safety-tested virus strains such as modified vaccinia virus Ankara (MVA) have made this versatile eukaryotic expression system even more attractive for basic and clinical research. Here, we report on easily obtaining recombinant MVA using stringent growth selection on rabbit kidney RK-13 cells. We describe the construction and use of new MVA vector plasmids that carry an expression cassette of the vaccinia virus host range gene, K1L, as a transient selectable marker. These plasmids allow either stable insertion of additional recombinant genes into the MVA genome or precisely targeted mutagenesis of MVA genomic sequences. Repetitive DNA sequences flanking the K1L gene were designed to remove the marker gene from the viral genome by homologous recombination under nonselective growth conditions. The convenience of this new selection technique is demonstrated by isolating MVA recombinants that produce green fluorescent protein and by generating MVA deletion mutants.

INTRODUCTION

Genetically altered vaccinia virus serves as a well-established expression system in the laboratory and has proven successful for the development of new candidate recombinant vaccines (9). The introduction of vector viruses based on the replication-deficient modified vaccinia virus Ankara (MVA) provided the capability for high-level gene expression while promising exceptional biological safety (15). Recombinant MVA has been successfully evaluated for vaccination against a variety of infectious diseases or cancer in animal models (1,4,12,17), and the first MVA vectors have now entered clinical investigation. While there is increasing demand for the evaluation of new viral constructs, the generation of MVA vectors is different from replication-competent recombinant vaccinia virus because of the growth deficiency of the virus and the diminished cytopathic effects.

Quick and easy methods for generating recombinant MVA are necessary to compare multiple candidate constructs. In addition, as the use of recombinant MVA vectors approaches clinical application, more accessible genetic engineering should enable the generation of virus vectors that contain multiple genomic insertions and modifications, offering possible advantages for specific prophylaxis or therapy. Previously, several protocols were used to isolate recombinant MVA, relying mainly on the co-production of reporter enzymes such as the *E. coli* β-galactosidase and β-glucuronidase, which allow screening of foci of MVA-infected cells by colorimetric assays (2,4,12, 15). Additional co-expression of the *E. coli gpt* gene encoding xanthine-guanine-phosphoribosyl-transferase has been shown to simplify purification of recombinant MVA by allowing selec-

tion for resistance against the antimicrobial agent, mycophenolic acid (11, 15). The staining procedures usually require an additional 12–24 h of tissue culture, supplementation of agar overlays and the use of chromogenic substrates and antibiotics. Here, we report a quicker and more convenient method for obtaining recombinant MVA using the vaccinia virus host range gene, K1L, as the selective growth marker in rabbit kidney RK-13 cells.

To generate conventional vaccinia virus vectors, host range selection systems were successfully established after reinsertion of K1L coding sequences into the genome of K1L-defective viruses (10,13). Another host range selection technique to generate recombinant vaccinia virus is based on the use of an engineered vaccinia virus mutant that lacks the essential vaccinia virus gene, D4R, and that can only be propagated in a complementing cell line producing the D4R-encoded viral enzyme. Stringent selection of recombinant viruses is achieved after stable reinsertion of the D4R gene that allows the rescue of fully replication-competent viruses (6). Previous characterization of the MVA host range defect demonstrated that the repair of vaccinia virus coding sequences affected by the major deletion II within the MVA genome, including the host range gene K1L, rescued MVA replication in RK-13 cells (8). The nonpermissive MVA infection of RK-13 cells results in an early block of viral replication characterized by impaired synthesis of intermediate and late viral RNAs. Stable coexpression of the K1L gene and a eukaryotic expression plasmid in RK-13 cells complemented the defective virus life cycle and at least partially restored MVA production (16). Here, we show that introducing the K1L gene into the viral genome can be used for the efficient selection of recombinant MVA. We describe the construction and use of MVA vector plasmids that carry a K1L expression cassette as a transient selectable marker. We have evaluated this system for generating recombinant MVA that produce green fluorescent protein (GFP) and MVA deletion mutants.

MATERIALS AND METHODS

Cells and Viruses

Primary chicken embryo fibroblasts (CEF), baby hamster kidney BHK-21 cells (ATCC CCL-10) and rabbit kidney RK-13 cells (ATCC CCL-37) were grown in minimal essential media (MEM) supplemented with 10% fetal calf serum (FCS). Cells were maintained in a humidified air-5% CO_2 atmosphere at 37°C. Vaccinia virus strain

MVA (cloned isolate F6) (1,4,8,15,16) was routinely propagated and titered by vaccinia virus-specific immunostaining in CEF to determine the number of infectious units/mL. Virus from 582nd CEF passage was used for this study.

Plasmid Constructions

The complete K1L coding sequence under transcriptional control of its authentic promoter was amplified by PCR as a 1100-bp DNA fragment from the

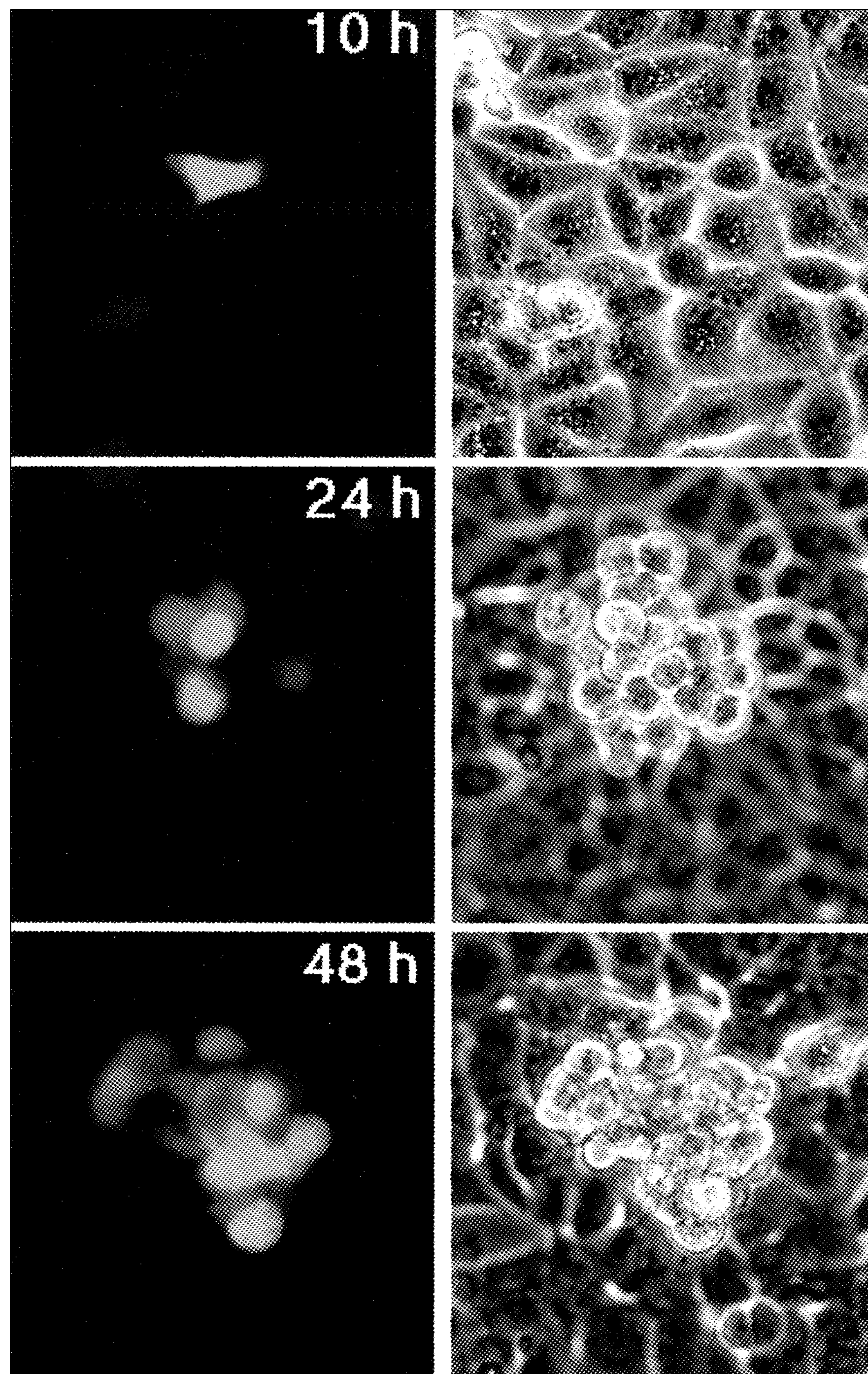

Figure 1. Microscopy of living RK-13 cells infected with MVA-gfp-K1L+. Cell monolayers were monitored using an environmental chamber mounted on an inverted Axiovert 135TV microscope. Images of an isolated focus of infected RK-13 cells taken by a 12-bit charge-coupled device camera at 10, 24 and 48 h after infection are shown. UV light images appear on the left, and phase-contrast images on the right.

genomic DNA of vaccinia virus strain Western Reserve (provided by Dr. B. Moss, LVD-NIH, Bethesda MD, USA) using the oligonucleotides K1L-5′-3 CAGCAGCCCGGGTGCGATAGCC-ATGTATCTACTAATCAG and K1L -3′-1 CAGCAGCCCGGGGGAAAT-CTATCTTATATACAC (sites for the restriction enzyme *Sma*I are underlined). To generate pIIIdHR-P7.5 and pIIIdHR-sP, the K1L expression cassette was cloned between flanking MVA-DNA sequences (flank1, flank2) of MVA transfer plasmid pIII (15) (Figure 2A). DNA sequences for either the natural vaccinia virus early/late promoter P7.5 (7) or the strong synthetic vaccinia virus early/late promoter, sP (3), were substituted. A repetitive 283-bp MVA-DNA sequence homologous to the 3′-end of MVA-DNA flanking sequence, flank1, in pIII was inserted for deletion of the K1L marker gene by homologous recombination. A 723-bp DNA fragment containing the *gfp* ORF was excised from pEGFP (Clontech Laboratories GmbH, Heidelberg, Germany) using the restriction endonucleases *Nco*I and *Not*I, treated with Klenow polymerase and inserted into the *Pme*I site of pIIIdHR-sP or pIIIdHR-P7.5 to obtain the *gfp* expression plasmids pIIIdHR-sP-gfp and pIIIdHR-P7.5-gfp.

pΔK1L was obtained by inserting the K1L expression cassette into plasmid pGEM-7Zf(±) (Promega, Madison, WI, USA) (Figure 3A). Two identical 216-bp PCR fragments derived from the 3′ coding sequence of *E. coli lac*Z gene were inserted as direct repeats on either side of the K1L expression cassette. Multiple cloning sites for restriction endonucleases (MCS1 and MCS2) were introduced adjacent to both repetitive sequences. The deletion plasmid pΔK1L-184R was constructed by introducing two DNA fragments that precisely flanked the nonessential MVA ORF 184R into MCS1 and MCS2, respectively. Flank184-1 consists of a 486-bp MVA-DNA sequence starting in the 5′-intergenic region of ORF 184R and ending at the start codon for translation of ORF 184R; flank184-2 is a 544-bp PCR fragment of MVA-DNA extending from the codon for 184R translation termination into the 3′-intergenic region of the 184R gene.

Generation of Recombinant MVA

Monolayers of 1×10^6 confluent

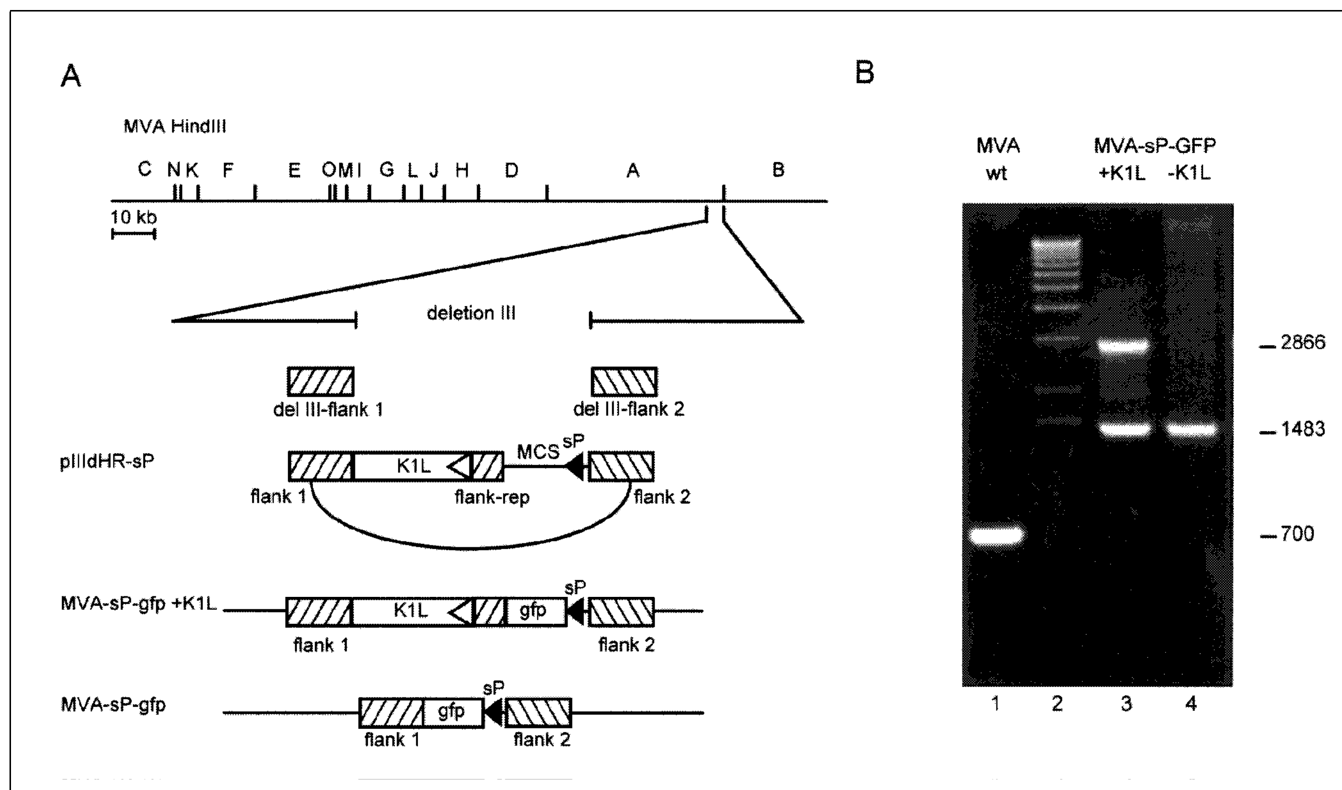

Figure 2. Generation of recombinant virus MVA-sP-gfp by transient K1L gene expression and growth selection in RK-13 cells. (A) Plasmid construction and insertion of recombinant genes. Schematic maps of the MVA genome (*Hin*dIII restriction map) and the vector plasmid pIIIdHRsP are shown on the top. Flank 1 and flank 2 refer to the MVA-DNA sequences that are essential to target insertion of foreign genes to the site of deletion III within the MVA genome. Flank-rep indicates the position of a 283-bp repetitive MVA-DNA fragment homologous to the right end of flank 1, which allows deletion of the K1L expression cassette by homologous recombination. Representations of the genomic structures of recombinant MVA-sP-gfp containing the *gfp* gene controlled by a vaccinia-specific synthetic early/late promoter (sP) and with (MVA-sP-gfp+K1L) or without (MVA-sP-gfp) the transient selectable marker gene K1L are shown at the bottom. (B) PCR analysis of recombinant viral DNA followed by agarose gel electrophoresis. Genomic DNA of wild-type MVA (wt, lane 1), recombinant MVA-sP-gfp containing the K1L marker gene (+K1L, lane 3) and final recombinant MVA-sP-gfp (-K1L, lane 4) served as template DNA for amplification of distinctive DNA fragments. Molecular weights were determined in comparison to the 1-kb ladder (Life Technologies, Rockville, MD, USA) (lane 2). Numbers on the right indicate the sizes (in bp) of the DNA fragments.

BHK-21 cells grown in 6-well tissue-culture plates (Corning, Corning, NY, USA) were infected with MVA at an MOI of 0.01. Ninety minutes after infection, the cells were transfected with 10 µg plasmid DNA/well using calcium phosphate (CellPhect Transfection Kit; Amersham Pharmacia Biotech, Freiburg, Germany) as recommended by the manufacturer. Forty-eight hours after infection, the cells were harvested, freeze-thawed three times and homogenized in a cup sonicator (Sonopuls HD 200, Bandelin, Germany). Tenfold serial dilutions (10^{-1} to 10^{-4}) of the harvested material in media were used to infect subconfluent monolayers of RK-13 cells grown in 6-well tissue-culture plates. After three days of incubation at 37°C, the foci of RK-13 cells infected with recombinant MVA were picked in a 20-µL volume by aspiration with an air-displacement pipet, transferred to microcentrifuge tubes containing 500 µL media and processed by freeze-thawing and sonication for subsequent infection of RK-13 cell monolayers. After the elimination of all parental MVA during passage in RK-13 cells, tenfold serial dilutions (10^{-1} to 10^{-6}) of the recombinant viruses were used for infection of subconfluent BHK-21 cells grown in 6-well tissue-culture plates (Corning). Well-separated foci of infected BHK-21 cells were harvested to isolate K1L-negative recombinant MVA.

PCR Analysis of Viral DNA

Genomic viral DNA was isolated from infected cells as described previously (5). Recombinant MVA were analyzed by PCR using oligonucleotides annealing within flanking regions of deletion III (MVA-III-5′: 5′-GAATGCACATACATAAGTACCGGCATCTCTAGCAGT-3′ and MVA-III-3′: 5′-CACCAGCGTCTACATGACGAGCTTCCGAGTTCC-3′), according to the following protocol: an initial step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 55°C for 40 s, 72°C for 3 min and a termination step at 72°C for 7 min.

Mutant MVA were analyzed by PCR using oligonucleotides annealing within the flanking regions, flank184-1 and -2, respectively (pair 1), or within flank184-1 and the coding region of 184R, respectively (pair 2). Reactions were performed according to the following protocol: an initial step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 52°C (pair 1) or 50°C (pair 2) for 40 s, 72°C for 3 min and a termination step at 72°C for 7 min.

Fluorescence Microscopy of Living Cells

Microscopy of living cells producing GFP was performed after infection of confluent 1×10^6 RK-13 cell monolayers grown in glass-bottom petri dishes (MatTek, Ashland, MA, USA) with recombinant MVA at an MOI of 0.0001. Dishes were analyzed by an inverted Axiovert 135TV research microscope (Zeiss, Oberkochen, Germany) with a mounted environmental chamber. Images were taken at 320-fold magnification with a cooled 12-bit charge-coupled device camera (Quantix®; Photometrics, Tuscon, AZ, USA) controlled by a Macintosh® PowerPC computer with IPLab® Spectrum 3.2 software.

Monitoring of MVA Growth on RK-13 Cells

Monolayers of 1×10^6 RK-13 cells grown in 6-well tissue-culture dishes (Corning) were infected with recombinant MVA at 1×10^5 infectious units/well or 1 infectious unit/well using a 1-mL volume for inoculation. After an adsorption period of 12 h at 37°C, fresh media were added to the inoculum, and cultures were incubated at 37°C for six days. Cytopathic effects were monitored daily by light microscopy.

RESULTS AND DISCUSSION

Our aim was to establish a host range selection system for quick isolation of recombinant MVA. Simultaneously, we wished to strictly conserve the well-characterized restricted host range of the virus. Therefore, we constructed new MVA vector plasmids, pIIIdHR-P7.5 and pIIIdHR-sP (Figure 2A), which target insertion of foreign genes precisely to the site of the major deletion III in the MVA genome. These vectors allow for transient K1L expression during growth selection and subsequent deletion of the host range marker gene

from the genome of viral vectors by intragenomic homologous recombination.

Compared with another successful protocol of transient marker gene expression using *E. coli lac*Z and *gpt* genes (11), our strategy is based on selective MVA growth in rabbit kidney RK-13 cells and does not require supplementation of chromogenic or selective substrates for isolating marker-free recombinant MVA. As a model gene, we inserted the coding sequence for GFP from the jellyfish *Aequorea victoria* and generated the expression plasmids pIIIdHR-sP-gfp and pIIIdHR-P7.5-gfp. After transfecting these plasmids into MVA infected BHK-21 cells, we obtained recombinant viruses that could be successfully isolated by passage in RK-13 cells. Selective growth of MVA-K1L+ recombinant viruses was recognized by the formation of an easily detectable cytopathic effect.

We observed the foci of virus-infected RK-13 cells as typical cell aggregations, first visible as early as 24 h after infection and continuously growing in size for at least three more days (Figure 1). During RK-13 passage, these aggregations were directly picked from the monolayer with no need for an agar overlay. Microscopic monitoring for GFP-fluorescent cells in aggregations formed after infection of RK-13 cell monolayers allowed convenient assessment of recombinant gene expression. During the first RK-13 passage generating recombinant MVA-sP-gfp, we could detect GFP synthesis in 27 out of 37 foci of infected cells, confirming the efficiency of selection. Furthermore, we found expression of the *gfp* gene driven by promoter sP detectable as early as 10 h after infection (Figure 1). After isolation of pure clonal recombinant viruses, MVA-sP-gfp and MVA-P7.5-gfp, during RK-13 passage, further plaque purification was carried out on BHK-21 cells to remove the K1L marker gene from the recombinant MVA genome. This was possible because MVA replication on BHK-21 cells does not require K1L gene function(s).

Genomic structures of recombinant viruses and the absence of contaminating nonrecombinant MVA were confirmed by PCR analysis of viral DNA (Figure 2B). We used oligonucleotides that anneal within the MVA-DNA flanking sequences, flank1 and flank2, to allow amplification of specific DNA fragments to differentiate recombinant and nonrecombinant MVA. The examination of viral DNA isolated from RK-13 cultures confirmed successful co-insertion of the K1L and GFP expression cassettes into the MVA genome. DNA fragments of 2866 bp comprising the K1L and GFP coding sequences were specifically amplified (Figure 2B, lane 3). As the K1L marker gene is flanked by repetitive sequences of MVA-DNA,

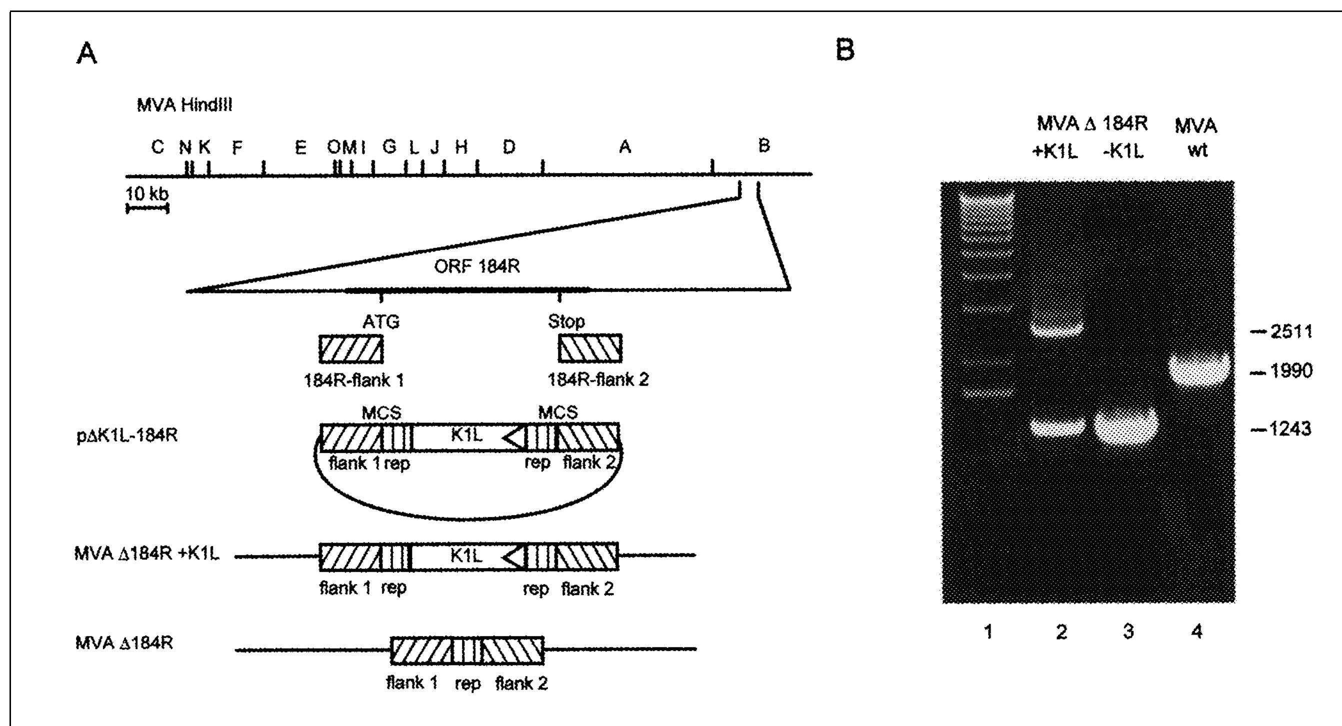

Figure 3. Targeted deletion of MVA gene sequences and PCR analysis of mutant virus genomes. (A) Deletion of nonessential MVA-DNA sequences using plasmid pΔK1L. A schematic *Hin*dIII restriction map of the MVA genome indicates the position of the target ORF 184R. Flanking sequences of MVA-DNA (184R-flank 1 and 184R-flank 2) were cloned into plasmid pΔK1L to allow deletion of ORF 184R by insertion of K1L coding sequences through homologous recombination. Two copies of identical *lac*Z-derived DNA fragments (rep) are located on either side of the K1L gene for subsequent removal of the selectable marker by homologous recombination. The target gene region of resulting MVA deletion mutants is shown at the bottom. MVA-Δ184R+K1L indicates mutant viral genomes transiently expressing the K1L gene. MVA-Δ184R refers to the mutant MVA that carry the rep DNA sequence to replace the 184R target gene. (B) PCR analysis of mutant MVA-DNA. Shown are amplified DNA fragments separated by agarose gel electrophoresis using the following template DNA: MVA-Δ184R+K1L (lane 2), MVA-Δ184R (lane 3), wild-type MVA (wt, lane 4); the 1-kb ladder served as molecular weight marker (lane 1). Numbers on the right indicate the sizes (in bp) of the DNA fragments.

it can be excised from the viral genome by homologous recombination during viral DNA replication. It was expected that this deletion mechanism would take place, at least to some extent, during selection in RK-13 cells. Correspondingly, we could also amplify 1483-bp DNA fragments specific for viral genomes containing the GFP expression cassette only (Figure 2B, lane 3). The PCR products obtained from the recombinant genomes were easily distinguished from a smaller 700-bp DNA fragment representing the deletion III-specific amplification product from nonrecombinant MVA-DNA (Figure 2B, lane 1). PCR analysis of recombinant viral DNA from BHK-21 cells revealed the 1483-bp DNA fragment as the only PCR product that confirmed the stable insertion of *gfp* gene sequences and demonstrated that the K1L expression cassette had been quickly deleted from all recombinant genomes after passage in these cells (Figure 2B, lane 4). The latter was confirmed by growth analysis on RK-13 cells. After infecting 10 monolayers of 1×10^6 cells with 10^5 infectious units of recombinant MVA-sP/monolayer, we failed to detect any cytopathic effect. Intact cell monolayers were maintained during the complete observation period. In contrast, we easily found foci of infected cells in 8 out of 10 RK-13 monolayers infected with as little as 1 infectious unit of another recombinant MVA which stably expresses the K1L host range gene (unpublished results).

Because of the ease of this new growth selection technique for recombinant MVA, we decided to take advantage of the K1L selectable marker in our continued efforts for improved genetic engineering of the MVA genome. Specifically, the precisely targeted deletion of original DNA sequences from the MVA genome could have several benefits: (*i*) the identification of nonessential gene regions to be used as additional insertion sites for recombinant genes; (*ii*) the removal of truncated gene sequences that could theoretically still enhance the large packaging capacity of the MVA genome; or (*iii*) the possibility to specifically inactivate viral gene products, to possibly improve immunogenicity of recombinant viruses.

The transfer plasmid pΔK1L was constructed for the specific deletion of target sequences within the MVA genome. It contains a functional copy of the vaccinia virus K1L gene under transcriptional control of its authentic promoter and two identical 216-bp PCR fragments derived from the *E. coli lac*Z gene as direct repeats on either side of the K1L expression cassette (Figure 3A). Adjacent to both repetitive sequences, we introduced multiple cloning sites for restriction endonucleases (MCS1 and MCS2) for convenient cloning of appropriate MVA-DNA sequences. The latter are chosen to selectively direct insertion of the K1L expression cassette to a given target site within the MVA genome that can result in deletion of nonessential DNA sequences from the MVA genome (Figure 3A). After isolation of the newly engineered MVA upon RK-13 growth selection, passage in BHK-21 cells should allow the deletion of the K1L marker gene from the viral genome by recombination of the co-inserted repetitive *lac*Z gene sequences.

To test the pΔK1L plasmid for MVA mutagenesis, we chose the nonessential but conserved MVA ORF 184R as a model gene to construct mutant MVA by a targeted gene knock-out. We generated deletion vector pΔK1L-184R by introducing two DNA fragments that flank the 184R ORF precisely on the 5′ and 3′ untranslated region into MCS1 and MCS2, respectively (Figure 3A). This design should allow the deletion of only the coding region of 184R and the conservation of intergenic regions that may contain essential regulatory sequences for the transcription of MVA genes adjacent to the target gene. Similar to the construction of recombinant viruses MVA-sP-gfp and MVA-P7.5-gfp, we generated mutant virus MVA-Δ184R after transfection of MVA-infected BHK-21 cells with pΔK1L-184R DNA and removed wild-type MVA by passage in RK-13 cells. Again, we monitored plaque purification of engineered MVA by PCR analysis. First, we used oligonucleotides that anneal to flank184-1 and flank184-2, respectively, and showed successful deletion of the 184R ORF. We could specifically amplify 2511 and 1243-bp DNA fragments from mutant genomes either containing or having deleted the K1L

marker gene sequences (Figure 3B, lane 2). In comparison, we obtained a 1990-bp fragment from wild-type MVA template DNA harboring full-length 184R sequences (Figure 3B, lane 4).

To further probe for absence of wild-type MVA, we performed another PCR using oligonucleotides that anneal to flank 1 and within the 184R ORF, and specifically generate a 975-bp DNA fragment in the presence of non-mutant template DNA (data not shown). After isolation of pure clonal MVA-Δ184R from RK-13 cells, we passaged the virus in BHK-21 cells to delete the K1L expression cassette from mutant genomes. Again, we could follow this process by amplifying a 1243-bp DNA fragment with primers annealing within flank184-1 and 184-2 (Figure 3B, lane 3). The successful generation of MVA-Δ184R demonstrates that the K1L-based growth selection can be used for the mutational modification of the MVA genome, for example, for deletion of nonessential MVA-DNA sequences. Note that even if the gene sequence targeted for deletion is essential for the MVA life cycle, viruses productively growing in RK-13 cells may be obtained. The complete vector plasmid can integrate into the viral genome through a single crossover event using either flanking MVA sequence of the shuttle vector (14). Due to high selective pressure, a virus resulting from such a single integration could carry the K1L selectable marker and grow in RK-13 cells, but still maintain the complete coding sequence of the target gene. Our PCR using oligonucleotides specific for the flanking target gene sequences (flank1 and flank2) would always generate an amplification product that would correspond in size to the wild-type target sequence. To further rule out a single crossover event, another PCR with one oligonucleotide primer annealing in the flanking target gene sequences and a second oligonucleotide specific for the plasmid backbone sequence could be performed.

In summary, we described (*i*) the construction of new MVA plasmid vectors containing the vaccinia virus host range gene K1L as a selectable marker, (*ii*) the stringent growth selection of K1L-expressing recombinant MVA through passage in RK-13 cells and (*iii*) the efficient removal of the K1L gene from the genome of recombinant MVA after passage in BHK-21 cells. Compared to previously established methodology, transient K1L-selection appears especially attractive because it offers the possibility for simple, positive dominant selection. Stringent isolation of recombinant or mutant MVA requires growth passage in well-established

standard cell lines. There is no need for supplementation of selective or chromogenic agents. This might be an advantage if the desired recombinant viruses are to be used for vaccine purposes or if particular genes are deleted to determine the specific effects on the phenotype of the mutant MVA. Moreover, the rapid loss of the K1L marker gene under nonselective growth conditions simplifies the task of generating multiple gene insertions or mutations in succession as it allows convenient reuse of the K1L-based selection procedure.

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