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Generation of a Complete Single-Gene Knockout Bacterial Artificial Chromosome Library of Cowpox Virus and Identification of Its Essential Genes

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Cowpox virus (CPXV) belongs to the genus *Orthopoxvirus* in the *Poxviridae* family. It infects a broad range of vertebrates and can cause zoonotic infections. CPXV has the largest genome among the orthopoxviruses and is therefore considered to have the most complete set of genes of all members of the genus. Since CPXV has also become a model for studying poxvirus genetics and pathogenesis, we created and characterized a complete set of single gene knockout bacterial artificial chromosome (BAC) clones of the CPXV strain Brighton Red. These mutants allow a systematic assessment of the contribution of single CPXV genes to the outcome of virus infection and replication, as well as to the virus host range. A full-length BAC clone of CPXV strain Brighton Red (pBRF) harboring the gene expressing the enhanced green fluorescent protein under the control of a viral late promoter was modified by introducing the *mrfp1* gene encoding the monomeric red fluorescent protein driven by a synthetic early vaccinia virus promoter. Based on the modified BAC (pBRFseR), a library of targeted knockout mutants for each single viral open reading frame (ORF) was generated. Reconstitution of infectious virus was successful for 109 of the 183 mutant BAC clones, indicating that the deleted genes are not essential for virus replication. In contrast, 74 ORFs were identified as essential because no virus progeny was obtained upon transfection of the mutant BAC clones and in the presence of a helper virus. More than 70% of all late CPXV genes belonged to this latter group of essential genes.

Cowpox virus (CPXV) belongs to the family *Poxviridae* and the genus *Orthopoxvirus*. While the prototype virus of the genus, the human pathogen variola virus, was declared eradicated in 1980, other members of the genus, including CPXV and monkeypox virus, still circulate within animal populations and cause zoonotic infections in Western Eurasia and Africa, respectively (1). CPXV is closely related to vaccinia virus (VACV) that was used as a heterotypic live virus vaccine against smallpox. Studies investigating the function of orthopoxvirus (OPV) genes have mainly been conducted using VACV (2–5), although most VACV strains are attenuated in many species. In addition, VACV lacks functional copies of several genes present in other OPVs due to loss or truncation of the respective ORFs compared to other species of the genus (6). Consequently, the function of a number of known or predicted OPV genes and their products remains unknown, and VACV seems not to be the ideal model for studying OPV gene function.

In contrast, CPXV has a number of advantages that make it a suitable model virus for studying OPV biology. With a size of ~220 kbp, it has the largest genome of all OPVs, ~30 kbp larger than that of VACV. In addition, CPXV possesses the most complete genome of all known OPVs in terms of number of genes (7, 8) and is considered to have the potential to evolve into new virus species with pathogenic potential comparable to that of more virulent viruses (9). CPXV can infect a broad range of domestic and wild animals, including bovines, elephants, primates, cats, and rodents, and can also cause zoonotic disease in humans (1). It has become a popular model for the study of poxvirus biology and pathogenesis, but comprehensive functional analysis of poxvirus genes requires targeted genome manipulations. Classical methods to modify poxvirus genomes relying on homologous recombination in vertebrate cells are laborious and time-consuming (10). Progeny viruses containing the desired modified genome are rare, because of low recombination efficiencies, which make necessary multiple rounds of plaque purification and sometimes even chemical selection or comprehensive screening protocols (10). Knockout mutants of genes important for virus replication might be difficult or almost impossible to purify because of their greatly impaired fitness. Moreover, mutant isolation might need multiple rounds of selection, even when complementing cell lines are used (11).

Bacterial artificial chromosome (BAC) technology is a powerful tool used to propagate and modify large genomic DNA fragments and has been used to clone an increasing number of different virus genomes, among them various poxviruses (12–16). Even after sequential introduction of six major deletions into a chorioallantois vaccinia virus Ankara BAC clone, no fortuitous mutations occurred (16). This underlines the suitability of the BAC technology for maintenance and modification of poxvirus genomes. Mutagenesis methods such as two-step markerless Red recombination (also referred to as "*en passant*" mutagenesis) allow efficient modification of BAC DNA in *Escherichia coli* ranging from single base pair mutation to the deletion or introduction of large pieces of DNA (17, 18). Red recombination only requires short homologous sequences (>28 bp) to insert foreign DNA into a target site, which makes the design and execution of mutagenesis relatively simple. For comparison,

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recombination in infected vertebrate cells requires homologous sequences of at least 200 bp in order to achive relatively high efficiencies (19).

In the present study, we constructed a knockout library of the CPXV strain Brighton Red (BR) genome, which was based on a full-length virus BAC clone termed pBRFseR. pBRFseR was constructed by introducing a red fluorescent marker (mRFP) into pBRF, a previously described full-length BAC clone (15). Thus, pBRFseR contains a mRFP driven by an early promoter and an enhanced green fluorescent protein (eGFP) under the control of a viral late promoter, both of which allowed us to monitor early and late gene expression of CPXV. We used Red recombination in *E. coli* to insert deletion cassettes containing stop codons, as well as a kanamycin resistance gene, which resulted in single-gene knockout mutants for each unique CPXV-BR open reading frame (ORF). After reconstitution of each of the mutant viruses, we identified different phenotypes and were able to categorize CPXV genes as essential for the transition to late gene expression, essential for the production of virus progeny despite the production of late viral proteins, or nonessential for viral replication in cell culture.

MATERIALS AND METHODS

Cell lines and viruses. All cell lines were cultivated at 37°C under a 5% CO_2 atmosphere. African green monkey cells Vero 76 (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) were maintained in Eagle's minimal essential medium with Earle's salts, 2.2 g of $NaHCO_3$/liter, and stable L-glutamine (MEM; Biochrom, Berlin, Germany) supplemented with 5% fetal bovine serum (FBS; Biochrom), 65 μg of penicillin G/ml, and 100 μg of streptomycin (AppliChem GmbH, Darmstadt, Germany)/ml. Primary chicken embryo cells (CEC) were prepared from 11-day-old embryonated specific-pathogen-free eggs (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany) according to standard procedures and cultured in MEM containing 10% FBS (Biochrom) and antibiotics as described above. Recombinant and mutant CPXV were propagated on Vero cells, whereas fowlpox virus (FWPV; Nobilis-PD, strain WP; Intervet, Boxmeer, Netherlands [kindly provided by D. Lüschow, Freie Universität, Berlin, Germany]) was amplified on CEC.

Reconstitution of infectious virus from BAC DNA. For virus reconstitution, 1×10^5 or 7×10^5 Vero cells seeded in one well of a 24- or 6-well plate, respectively, were transfected with ~2 μg of purified BAC DNA using 1 to 4 μl of FuGENE HD transfection reagents (Promega, Mannheim, Germany) according to the manufacturer's instructions. Transfected cultures were infected with 20 to 500 PFU of FWPV at 2 h after transfection. Virus reconstitution was monitored using an Axiovert 100 fluorescence microscope (Carl Zeiss, Jena, Germany) by screening for fluorescent early and late gene expression markers. Images were taken from 48 to 240 h after transfection using an AxioCam MRm charge-coupled device camera (Zeiss). Image processing was performed with the AxioVision 4.8.2 software package (Zeiss). Elimination of residual helper virus was achieved by passaging the reconstituted viruses three times on Vero cells, which are nonpermissive for FWPV. Between individual passages, infected cells were lysed by freeze-thawing the cultures twice at −70°C. Confluent monolayers in 24- or 6-well plates were infected with 1 to 10 μl of freeze-thaw cell lysate from the previous passage. Monitoring of virus replication and imaging of single virus plaques was performed as described above. Mutant BAC clones, which could not be reconstituted on the first attempt, were used for repeated transfections on Vero cells with FWPV and on CEC using Shope fibroma virus (SFV; Merial, Lyon, France) as a helper virus.

Generation of plasmid pACAA. For the construction of plasmid pACAA, the vector pACYC177 (New England BioLabs, Frankfurt, Germany) was amplified via inverse PCR using the primers pAAfw and pAArv (see Table S1 in the supplemental material [http://www.vetmed.fu-berlin.de/en/einrichtungen/institute/we05/cowpox]). This resulted in the deletion of 205 bp from the plasmid and insertion of a bacterial promoter and the kanamycin resistance gene (*aphAI*), together with a 9-bp sequence (GCCGCGTGA) that codes for two alanines and a stop codon, while also containing a PaeI restriction site. After digestion of the PCR product with DpnI to eliminate template vector, the DNA was cleaved with PaeI and religated resulting in the vector pACAA. This plasmid was used as a template for the generation of the knockout library.

Generation of dual marker CPXV-BR BAC clone pBRFseR. To generate transfer vector pEP-MVA-dVI-PK1L-mRFP (18), the gene encoding monomeric red fluorescence protein 1 (*mrfp1*) (20) was excised from plasmid pEP-ExpRFP1-in by using BamHI and SacI. The linear 763-bp fragment was inserted into the respective restriction sites in pEP-MVA-dVI-PK1L (Dai LianPan and Ingo Drexler, unpublished data).

Insertion of the PK1L-*mRFP1* expression cassette into pBRF (15) was performed by two-step *en passant* Red recombination as described previously (17, 18) (Fig. 1). Briefly, the PK1L-*mRFP1-aphAI* fragment was amplified from pEP-MVA-dVI-PK1L-mRFP by using the primers mRFPfw and mRFPrv (see Table S1 in the supplemental material) and electroporated into *E. coli* strain GS1783 (17) harboring the pBRF BAC clone. Insertion of the PK1L-*mRFP1* expression cassette into pBRF resulted in pBRFeR. In a second *en passant* mutagenesis procedure, the K1L promoter of the PK1L-*mRFP1* cassette in pBRFeR was replaced by the previously published synthetic early promoter ($P_{syn7.5}$) (21). For this purpose, the *aphAI*-I-SceI fragment from plasmid pEP-kanS was amplified with the primers syn7.5fw and syn7.5rv (see Table S1 in the supplemental material) and used for *en passant* recombination exactly as described earlier (18). All BAC clones described above were verified by restriction fragment length polymorphism (RFLP) analysis and sequencing of the insertion site (data not shown).

BAC mutagenesis. Knockout BAC clones were generated by inserting PCR-derived marker cassettes into selected loci by one-step Red recombination (17) (Fig. 2). All knockout BAC clones are listed in Table 1. Knockout BAC clones were named according to the respective ORF deleted. For example, the CPXV010 deletion mutant was named pBRFseR_d10. PCR primers were designed to amplify the *aphAI* cassette from recombinant plasmid pACAA. Besides the marker cassette, PCR fragments contained at each end 40 bp of sequences that were homologous to the target locus in the CPXV BR sequence. The resulting PCR products were inserted into pBRFseR by conventional Red recombination, ultimately resulting in interruption of all predicted ORFs in CPXV (Table 1 and see Table S2 in the supplemental material [http://www.vetmed.fu-berlin.de/en/einrichtungen/institute/we05/cowpox]). Recombination was performed in *E. coli* strain GS1783 by electroporation of PCR products into GS1783 harboring pBRFseR. Bacteria were spread on LB agar plates containing 35 μg of chloramphenicol/ml and 35 μg of kanamycin/ml (Roth, Karlsruhe, Germany) to select for clones containing the insertion cassette.

Knockout BAC verification. For each of the knockout mutants, bacterial colonies resistant to chloramphenicol and kanamycin were selected and BAC DNA was extracted by alkaline lysis (22). For RFLP analysis, BAC DNA was cleaved with selected restriction enzymes and separated by 0.8% agarose gel electrophoresis for 16 h at 75 V in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8.4]). Each individual mutant BAC DNA was tested for the correct RFLP with at least two different restriction enzymes (see Table S3 in the supplemental material [http://www.vetmed.fu-berlin.de/en/einrichtungen/institute/we05/cowpox]). To confirm the in-frame insertion of the premature stop codon, PCR primers covering the original start and new stop codon of the respective target ORFs were designed for all mutants (see Table S3 in the supplemental material). PCR products overlapping with the modified loci were checked by agarose gel electrophoresis, purified using the GF-1 AmbiClean (PCR & Gel) nucleic acid extraction kit (Vi-

FIG 1 Insertion of a fluorescent marker for viral early gene expression into the mini-F region of pBRF. The PK1L-*mRFP1*-*aphAI* fragment was amplified from transfer vector pEP-MVA-dVI-PK1L-mRFP using the primers mRFPfw and mRFPrv. The cassette was integrated downstream of the existing late gene expression marker (eGFP) by *en passant* mutagenesis, resulting in pBRFeR. In a second *en passant* mutagenesis, the K1L promoter was exchanged for a previously published synthetic early promoter (Psyn7.5), resulting in pBRFseR. Dashed lines indicate recombination events between homologous sequences.

vantis Technologies, Subang Jaya, Malaysia) and sequenced to verify the correct insertion of the marker cassette.

Bioinformatics analysis. Searches for homologous poxvirus sequences, as well as prediction of gene expression kinetics and function of encoded proteins, were performed using the NCBI BLAST and the VectorNTI 9.1 software package (Invitrogen, Darmstadt, Germany) and were based on OPV sequences available at the Poxvirus Bioinformatics Resource Center (PBRC; http://www.poxvirus.org) and GenBank (23). Predicted incorporation of CPXV proteins into the virion was based on information for homologous proteins of VACV (7, 24–26).

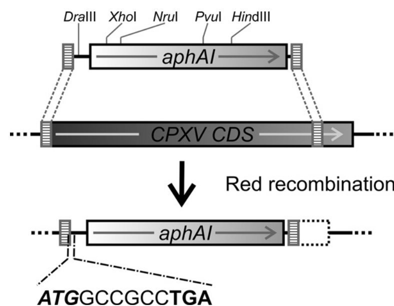

FIG 2 Schematic illustration of the generation of CPXV knockout mutants using Red recombination in *E. coli*. PCR fragments containing a kanamycin resistance gene *aphAI* and a sequence coding for two alanines (GCCGCG), followed by a stop codon (TGA) were amplified from plasmid pACAA. Homologous flanking sequences (40 bp) were added through 5′ overhangs of each PCR primer. PCR products were inserted into the target genes of the CPXV sequence by Red recombination. Restriction sites in PCR products for DraIII, HindIII, NruI, PvuI, and XhoI are indicated.

RESULTS AND DISCUSSION

Generation of the dual marker CPXV-BR BAC clone pBRFseR. We previously cloned the CPXV-BR genome as a BAC by inserting the mini-F harboring the *egfp* gene under the control of a viral late promoter into the locus of the nonessential thymidine kinase (*TK*, *CPXV105*) (15). In order to be able to monitor early and late viral gene expression in infected cells, we introduced the *mrfp1* gene under the control of a synthetic early promoter, based on the 7.5-kDa VACV promoter, into the recombinant parental clone pBRF. Since early gene promoters tend to be weak and homology to existing sequences in the BAC might have led to genetic instability, we chose a synthetic early promoter, optimized for strong expression (21). The early marker expression cassette was inserted into the mini-F region of the BAC by two-step *en passant* Red mutagenesis.

Successful integration resulting in the dual marker pBRFseR

TABLE 1 CPXV-BR ORFs, mutant BACs, and reconstitution

CPXV-BR gene	VACV-COP homolog(s)	Function of VACV homologs[a]	Mutant BAC	Reconstitution	Conservation[b]
CPXV001		Unknown			
CPXV002		Unknown			
CPXV003	C23L	Chemokine binding protein			
CPXV004		Unknown			
CPXV005	C22L	Tumor necrosis factor receptor			
CPXV006	C19L	Ankyrin			
CPXV007		Unknown			
CPXV008	C17L	Ankyrin			
CPXV009	C16L	Unknown			
CPXV010	N2L	Alpha-amanitin sensitivity protein	pBRFseR d10	Yes	No
CPXV011		Ankyrin			
CPXV012		Unknown	pBRFseR d12	Yes	No
CPXV013		Kelch like	pBRFseR d13	Yes	No
CPXV014f	C22L, B28R	Tumor necrosis factor receptor	pBRFseR d14	Yes	No
CPXV015f		Unknown	pBRFseR d15	Yes	No
CPXV016		Ankyrin			
CPXV017		Ankyrin			
CPXV018		Unknown	pBRFseR d18	Yes	No
CPXV019		Ankyrin			
CPXV020		Unknown	pBRFseR d20	Yes	No
CPXV021	C11R	Epidermal growth factor	pBRFseR d21	Yes	No
CPXV022	C10L, C4L	Interleukin-1 receptor antagonist	pBRFseR d22	Yes	No
CPXV023		Ubiquitin ligase/host defense modulator protein	pBRFseR d23	Yes	No
CPXV024		Interleukin-18 binding protein	pBRFseR d24	Yes	No
CPXV025		Ankyrin host range protein			
CPXV026		Unknown	pBRFseR d26	Yes	No
CPXV027	C9L	Ankyrin			
CPXV028	C8L	Unknown	pBRFseR d28	Yes	No
CPXV029	C7L	Host range virulence factor	pBRFseR d29	Yes	No
CPXV030	C6L	Unknown	pBRFseR d30	Yes	No
CPXV031f	C5L	Unknown	pBRFseR d31	Yes	No
CPXV032	C5L	Unknown	pBRFseR d32	Yes	No
CPXV033	C10L, C4L	Interleukin-1 receptor antagonist	pBRFseR d33	Yes	No
CPXV034	C3L	Complement binding protein (secreted)	pBRFseR d34	Yes	No
CPXV035	C2L	Kelch-like protein	pBRFseR d35	Yes	No
CPXV036	C1L	Unknown	pBRFseR d36	Yes	No
CPXV037	N1L	Virokine/NF-κB inhibitor	pBRFseR d37	Yes	No
CPXV038	N2L	Alpha amanitin sensitivity protein	pBRFseR d38	Yes	No
CPXV039	M1L	Ankyrin			
CPXV040	M2L	NF-κB inhibitor	pBRFseR d40	Yes	No
CPXV041	K1L	Ankyrin and NF-κB inhibitor			
CPXV042	C12L, K2L, B13R, B14R	"Serpin 1,2,3"	pBRFseR d42	Yes	No
CPXV043	K3L	Interferon resistance and eIF2 alpha-like PKR inhibitor	pBRFseR d43	Yes	No
CPXV044	K4L	Nicking/joining enzyme	pBRFseR d44	Yes	No
CPXV045	K5L, K6L	Monoglyceride lipase (putative)	pBRFseR d45	Yes	No
CPXV046	K7R	Unknown	pBRFseR d46+47	Yes	No
CPXV047	Pseudo?[c]	Unknown			
CPXV048	F1L	Apoptosis inhibitor (associated with mitochondria)	pBRFseR d48	Yes	No
CPXV049	F2L	dUTPase	pBRFseR d49	Yes	No
CPXV050	F3L	Kelch-like protein	pBRFseR d50	Yes	No
CPXV051	F4L	Ribonucleotide reductase small subunit	pBRFseR d51	Yes	No
CPXV051A	Pseudo?	Unknown			
CPXV052	F5L	Membrane protein (36 kDa)	pBRFseR d52	Yes	No
CPXV053	F6L	Unknown	pBRFseR d53	Yes	No
CPXV054	F7L	Unknown	pBRFseR d54	Yes	No
CPXV055	F8L	Cytoplasmic protein	pBRFseR d55	Yes	No
CPXV056	F9L	Disulfide bond formation pathway protein	pBRFseR d56	No	P
CPXV057	F10L	Serine/threonine kinase	pBRFseR d57+58	No	P

(Continued on following page)

TABLE 1 (Continued)

CPXV-BR gene	VACV-COP homolog(s)	Function of VACV homologs[a]	Mutant BAC	Reconstitution	Conservation[b]
CPXV058	F_ORF_D Pseudo?	Unknown			
CPXV059	F11L	Unknown	pBRFseR d59	Yes	No
CPXV060	F12L	IEV-associated protein	pBRFseR d60	Yes	C
CPXV061	F13L	EEV phospholipase	pBRFseR d61	Yes	C
CPXV062	F14L	Unknown	pBRFseR d62	Yes	No
CPXV063		Unknown	pBRFseR d63	Yes	No
CPXV064	F15L	Unknown	pBRFseR d64	Yes	C
CPXV065	F16L	Unknown	pBRFseR d65	Yes	No
CPXV066	F17R	DNA binding phosphoprotein	pBRFseR d66	No	C
CPXV067	E1L	Poly(A) polymerase large subunit	pBRFseR d67	No	P
CPXV068	E2L	Unknown	pBRFseR d68	Yes	C
CPXV069	E3L	Interferon resistance and PKR inhibitor	pBRFseR d69	Yes	No
CPXV070	E4L	RNA polymerase 30 subunit	pBRFseR d70	No	C
CPXV071	E5R	Virosome component protein	pBRFseR d71	Yes	No
CPXV072	E6R	required for the formation of immature virions	pBRFseR d72	No	P
CPXV073	E7R	EEV myristylated soluble protein	pBRFseR d73	Yes	No
CPXV074	E8R	Endoplasmic reticulum localized membrane protein	pBRFseR d74	Yes	C
CPXV075	E9L	DNA polymerase	pBRFseR d75	No	P
CPXV076	E10R	Disulfide bond formation pathway protein	pBRFseR d76	No	P
CPXV077	E11L	Virion core protein	pBRFseR d77	No	No
CPXV078	O1L	Unknown	pBRFseR d78	Yes	No
CPXV078A	Pseudo?	Unknown			
CPXV079	O2L	Glutaredoxin 1	pBRFseR d79	Yes	No
CPXV080	I1L	DNA binding protein	pBRFseR d80	No	C
CPXV081	I2L	Unknown	pBRFseR d81	No	C
CPXV082	I3L	DNA binding phosphoprotein	pBRFseR d82	No	C
CPXV083	I4L	Ribonucleotide reductase large subunit	pBRFseR d83	Yes	No
CPXV084	I5L	IMV protein	pBRFseR d84	Yes	C
CPXV085	I6L	Telomere binding protein	pBRFseR d85	Yes	C
CPXV086	I7L	Virion core protease	pBRFseR d86	No	P
CPXV087	I8R	RNA helicase/NPH-II	pBRFseR d87	No	P
CPXV088	G1L	Metalloprotease (predicted)	pBRFseR d88	No	P
CPXV089	G3L	virus fusion complex	pBRFseR d89	No	C
CPXV090	G2R	Viral late transcription factor	pBRFseR d90	No	C
CPXV091	G4L	Glutaredoxin 2	pBRFseR d91	No	C
CPXV092	G5R	Unknown	pBRFseR d92	No	P
CPXV093	G5.5R	RNA polymerase 7 subunit	pBRFseR d93	No	C
CPXV094	G6R	Unknown	pBRFseR d94	Yes	P
CPXV095	G7L	Virion assembly protein	pBRFseR d95	No	C
CPXV096	Pseudo?	Unknown			
CPXV097	G8R	Viral late transcription factor 1	pBRFseR d96+97	No	C
CPXV098	G9R	Entry fusion complex protein	pBRFseR d98	No	P
CPXV099	L1R	IMV myristylated membrane protein	pBRFseR d99	No	P
CPXV100	L2R	Crescent formation	pBRFseR d100	No	C
CPXV101	L3L	Internal virion protein	pBRFseR d101	No	P
CPXV102	L4R	Core package and transcription protein	pBRFseR d102	No	P
CPXV103	L5R	IMV entry and fusion protein	pBRFseR d103	No	P
CPXV104	J1R	Virion morphogenesis protein	pBRFseR d104	No	C
CPXV105	J2R	Thymidine kinase			
CPXV106	J3R	Poly(A) polymerase small subunit	pBRFseR d106	No	P
CPXV107	J4R	RNA polymerase 22 subunit	pBRFseR d107	No	C
CPXV108	J5L	Unknown membrane protein	pBRFseR d108	Yes	P
CPXV109	J6R	RNA polymerase 147 subunit	pBRFseR d109	No	P
CPXV110	H1L	Serine/Tyrosine phosphatase	pBRFseR d110	Yes	C
CPXV111	H2R	Entry and cell to cell fusion protein	pBRFseR d111	No	P
CPXV112	H3L	IMV heparin binding surface protein	pBRFseR d112	Yes	P
CPXV113	H4L	RNA polymerase associated protein RAP94	pBRFseR d113	No	P
CPXV114	H5R	Viral late transcription factor 4	pBRFseR d114	No	C
CPXV115	H6R	Topoisomerase type I	pBRFseR d115	Yes	P

(Continued on following page)

TABLE 1 (Continued)

CPXV-BR gene	VACV-COP homolog(s)	Function of VACV homologs[a]	Mutant BAC	Reconstitution	Conservation[b]
CPXV116	Pseudo?	Unknown			
CPXV117	H7R	Crescent formation	pBRFseR d117+116	No	C
CPXV118	D1R	Large capping enzyme	pBRFseR d118	No	P
CPXV119	D2L	Virion core	pBRFseR d119+119A	No	C
CPXV119A	D_ORF_B	Unknown			
CPXV120	D3R	Virion core protein	pBRFseR d120	No	C
CPXV121	D4R	Uracil DNA glycosylase	pBRFseR d121	No	P
CPXV122	D5R	NTPase and DNA replication protein	pBRFseR d122	No	P
CPXV123	D6R	Viral early transcription factor small subunit	pBRFseR d123	No	P
CPXV124	D7R	RNA polymerase 18 subunit	pBRFseR d124	No	P
CPXV125	D8L	Carbonic anhydrase	pBRFseR d125	Yes	No
CPXV126	D9R	NTP-PPH containing mutT motif	pBRFseR d126	Yes	C
CPXV127	D10R	NPH-PPH RNA levels regulator containing mutT motif	pBRFseR d127	Yes	P
CPXV128	D11L	Helicase NPH-I	pBRFseR d128	No	P
CPXV129	D12L	Small capping enzyme	pBRFseR d129+130	No	P
CPXV130	Pseudo?	Unknown			
CPXV131	D13L	Virion coat protein rifampin resistance	pBRFseR d131	No	P
CPXV132	A1L	Viral late transcription factor 2	pBRFseR d132	No	P
CPXV133	A2L	Viral late transcription factor 3	pBRFseR d133	No	P
CPXV134	A2.5L	Thioredoxin like protein	pBRFseR d134	No	C
CPXV135	A3L	P4b precursor	pBRFseR d135	No	P
CPXV136	A4L	Core protein	pBRFseR d136	Yes	C
CPXV137	A5R	RNA polymerase 19 subunit	pBRFseR d137	No	P
CPXV138	A6L	Virion morphogenesis protein	pBRFseR d138	No	C
CPXV139	A7L	Viral early transcription factor large subunit	pBRFseR d139	No	P
CPXV140	A8R	Viral intermediate transcription factor 3	pBRFseR d140	No	C
CPXV141	A9L	Membrane protein	pBRFseR d141	No	P
CPXV142	A10L	P4a precursor	pBRFseR d142	No	P
CPXV143	A11R	Membrane formation protein	pBRFseR d143	No	P
CPXV144	A12L	Structural protein	pBRFseR d144	No	C
CPXV145	A13L	Virion maturation protein	pBRFseR d145	No	C
CPXV146	A14L	IMV membrane protein (phosphorylated)	pBRFseR d146	No	C
CPXV147	A14.5L	IMV virulence factor (membrane protein)	pBRFseR d147	Yes	C
CPXV148	A15L	Unknown	pBRFseR d148	No	C
CPXV149	A16L	Entry and cell-to-cell fusion protein (myristilated)	pBRFseR d149	No	P
CPXV150	A17L	IMV membrane protein phosphorylated	pBRFseR d150	No	C
CPXV151	A18R	DNA helicase	pBRFseR d151	No	P
CPXV152	A19L	Unknown	pBRFseR d152+152A	No	C
CPXV152A	Pseudo?	Unknown			
CPXV153	A21L	Entry and cell-to-cell fusion protein	pBRFseR d153	No	P
CPXV154	A20R	DNA processivity factor	pBRFseR d154	No	C
CPXV155	A22R	Holliday junction resolvase	pBRFseR d155	Yes	P
CPXV156	A23R	Viral intermediate transcription factor 3 (45-kDa subunit)	pBRFseR d156	No	P
CPXV157	A24R	RNA polymerase 132 subunit	pBRFseR d157	No	P
CPXV158	A25L	A-type inclusion protein	pBRFseR d158	Yes	No
CPXV159	A26L	P4c precursor	pBRFseR d159	Yes	No
CPXV160	Pseudo?	Unknown			
CPXV161	A26L	P4c precursor	pBRFseR d161	Yes	No
CPXV162	A27L	Fusion protein	pBRFseR d162	Yes	No
CPXV163	A28L	IMV virus entry (membrane protein)	pBRFseR d163	No	P
CPXV164	A29L	RNA polymerase 35 subunit	pBRFseR d164	No	P
CPXV165	A30L	Virion morphogenesis protein	pBRFseR d165	No	C
CPXV166	A31R	Unknown	pBRFseR d166	Yes	No
CPXV167	A32L	DNA packaging and ATPase protein	pBRFseR d167	No	P
CPXV168	A33R	EEV glycoprotein	pBRFseR d168	Yes	No
CPXV169	A34R	EEV C-type lectin-like protein	pBRFseR d169	Yes	C
CPXV170	Pseudo?	Unknown	pBRFseR d170+171	Yes	No
CPXV171	A35R	Unknown			

(Continued on following page)

TABLE 1 (Continued)

CPXV-BR gene	VACV-COP homolog(s)	Function of VACV homologs[a]	Mutant BAC	Reconstitution	Conservation[b]
CPXV172	A36R	IEV specific	pBRFseR d172	Yes	No
CPXV173	A37R	Unknown	pBRFseR d173	Yes	No
CPXV174		Unknown	pBRFseR d174	Yes	No
CPXV175	A38L	CD47-like protein	pBRFseR d175	Yes	No
CPXV176	A39R	Semaphorin	pBRFseR d176	Yes	No
CPXV177	A40R	Lectin homolog	pBRFseR d177	Yes	No
CPXV178	A41L	Secreted virulence factor	pBRFseR d178	Yes	No
CPXV179	A42R	Profilin homolog	pBRFseR d179	Yes	No
CPXV180	A43R	Membrane glycoprotein class I	pBRFseR d180	Yes	No
CPXV181		Unknown	pBRFseR d181	Yes	No
CPXV182	A44L	Hydroxysteroid dehydrogenase	pBRFseR d182	Yes	No
CPXV183	A45R	Superoxide dismutase-like protein	pBRFseR d183	Yes	No
CPXV184	A46R	Interleukin-1 signaling inhibitor	pBRFseR d184	Yes	No
CPXV185	A47L	Unknown	pBRFseR d185	Yes	No
CPXV186	A48R	Thymidylate kinase	pBRFseR d186	Yes	No
CPXV187	A49R	Phosphotransferase anion transport protein (putative)	pBRFseR d187	Yes	No
CPXV188	A50R	DNA ligase	pBRFseR d188	Yes	No
CPXV189	A51R	Unknown	pBRFseR d189	Yes	No
CPXV190	A52R	Intracellular TLR and interleukin-1 signaling inhibitor	pBRFseR d190	Yes	No
CPXV191	A53R, A_ORF_T	Tumor necrosis factor receptor (CrmC)	pBRFseR d191	Yes	No
CPXV192	Pseudo?	Unknown			
CPXV193	A55R	Kelch-like protein	pBRFseR d193	Yes	No
CPXV194	A56R	Hemagglutinin	pBRFseR d194	Yes	No
CPXV195	A57R	Guanylate kinase	pBRFseR d195	No	No
CPXV196	B1R	Serine/threonine kinase	pBRFseR d196	Yes	No
CPXV197	B2R; B3R	Schlafen	pBRFseR d197	Yes	No
CPXV198	B4R	Ankyrin			
CPXV199	B5R	EEV complement control protein	pBRFseR d199	Yes	No
CPXV200	B6R	Unknown	pBRFseR d200	Yes	No
CPXV201	B7R	Virulence factor (endoplasmic reticulum associated)	pBRFseR d201	Yes	No
CPXV202	B8R	Interferon gamma receptor	pBRFseR d202	Yes	No
CPXV203	B9R	Virulence factor	pBRFseR d203	Yes	No
CPXV204	B10R	Kelch-like protein	pBRFseR d204	Yes	No
CPXV205	B11R	Unknown	pBRFseR d205	Yes	No
CPXV206	B12R	Serine/threonine kinase	pBRFseR d206	Yes	No
CPXV207	C12L, K2L, B13R, B14R	"Serpin 1,2,3"			
CPXV208	C16L, B15R, B22R	Unknown	pBRFseR d208	Yes	No
CPXV209	B16R	Interleukin-1β receptor	pBRFseR d209	Yes	No
CPXV210	B17L	Unknown	pBRFseR d210	Yes	No
CPXV211	B18R	Ankyrin			
CPXV212	B19R	Alpha/beta interferon receptor	pBRFseR d212	Yes	No
CPXV213	B20R	Ankyrin			
CPXV214	Pseudo?	Unknown			
CPXV215		Kelch-like protein	pBRFseR d214+215	Yes	No
CPXV216	C12L, K2L, B13R, B14R	Unknown	pBRFseR d216 (ATG)	Yes	No
CPXV217	C12L, K2L, B13R, B14R	"Serpin 1,2,3"	pBRFseR d217	Yes	No
CPXV218	C14L, C13L	Unknown	pBRFseR d218	Yes	No
CPXV219		Surface glycoprotein	pBRFseR d219	Yes	No
CPXV220	C21L, C20L, C19L, B25R, B26R, B27R	Ankyrin			
CPXV221	CrmD	Tumor necrosis factor receptor (CrmD)	pBRFseR d221	Yes	No

[a] Information was obtained from the PBRC.
[b] C, gene families conserved in chordopoxviruses; P, gene families conserved in poxviruses (32).
[c] Pseudo?, pseudogene (according to PBRC).

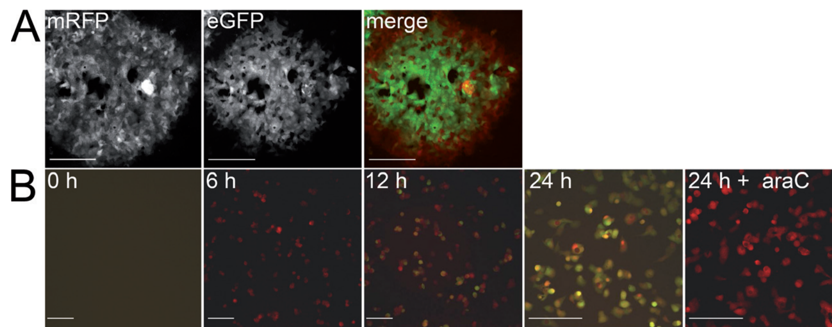

FIG 3 Detection of dual marker expression in infected cell culture. (A) Reconstitution of vBRFseR on Vero cells using FWPV as a helper virus. Expression of both mRFP and eGFP can be detected upon reconstitution. (B) Expression of mRFP and eGFP during infection. mRFP can be detected at 6 h p.i.; eGFP can be detected at 12 h p.i.; araC can block eGFP but not mRFP expression in infected cells, as evidenced by the expression of mRFP only. Scale bars, 200 μm.

BAC clone was checked by RFLP analysis and sequencing (data not shown). Upon reconstitution and serial virus passage, we obtained fully replicating virus (vBRFseR) expressing both the red and green fluorescent proteins (Fig. 3A). We performed time course experiments to test the kinetics of the expression of both fluorescent markers. Vero cells were infected with vBRFseR using a multiplicity of infection (MOI) of 1. The early red and the late green fluorescence signals were readily detectable at 6 and 12 h postinfection (p.i.), respectively.

Furthermore, addition of 50 μg of 1-β-D-arabinofuranosyl cytidine (araC)/ml to the cell culture did not affect expression of the early mRFP marker, while completely blocking expression of the late eGFP marker (Fig. 3B). This clearly showed that the newly introduced marker was indeed expressed in the early phase of viral replication, whereas the eGFP marker was only expressed in late stages of the replication cycle after successful replication of viral DNA (Fig. 3).

Targeted knockout of all unique CPXV-BR ORFs. The overall aim of the present study was to generate single knockout mutants for each of the 216 unique CPXV ORFs representing ORF *CPXV010* to ORF *CPXV221*. We did not include genes present in the terminal inverted repeats (TIR; ORFs *CPXV001* to *CPXV009* and *CPXV222* to *CPXV229*). Thirteen of the 216 unique ORFs were determined to be pseudogenes (according to the PBRC). Knockout mutants of all 16 ankyrin repeat protein-encoding genes, including 12 unique genes and the 4 ORFs in the TIR regions of the genome, were generated previously (B. K. Tischer, unpublished data). The thymidine kinase encoding gene *CPXV105* is interrupted by the mini-F replicon, and a BAC-based mutant of *CPXV207* coding for CrmA was described previously (15, 27). The six predicted kelch-like protein encoding genes were deleted by *en passant* mutagenesis for another study (Tischer, unpublished; see Fig. S1 in the supplemental material [http://www.vetmed.fu-berlin.de/en/einrichtungen/institute/we05/cowpox]). In the case of gene *CPXV216*, which is short and in close proximity to gene *CPXV217*, the start codon was replaced by *en passant* mutagenesis to avoid an interruption of the promoter of *CPXV217*.

We generated the knockout mutants of the remaining 182 ORFs by inserting the engineered PCR-derived bacterial selection marker using classical Red recombination (see Materials and Methods). We ensured disruption of the targeted ORF by positioning the bacterial selection marker such that it would certainly interrupt viral gene expression of the respective CPXV ORF and replacing between 200 and 1,000 bp of each gene depending on the respective length. Further, the insertions were targeted to be immediately after the start codon and an in-frame stop codon was added to avoid the production of larger truncated proteins, which could still be functional. Since poxvirus genes usually contain multiple start codons, we analyzed every ORF for additional downstream start codons and deleted the first two in-frame AUG codons. For example, the vaccinia virus E3L ORF can encode two functional proteins, p19 and p25, by initiating translation at its first or second AUG codon (28), and this gene was inactivated according to the outlined principle.

The knockout strategy described above would lead to double deletions in the case of overlapping ORFs, which occur frequently in poxvirus genomes. In CPXV-BR, for example, *CPXV036* and *CPXV037* overlap head to tail, with the promoter of *CPXV036* located in the coding sequence of *CPXV037*. Thus, deletion of *CPXV037* may lead to the simultaneous abrogation of *CPXV036* expression. In this and similar cases, we avoided interruption of the promoter of the downstream gene or damaging the integrity of the upstream gene by shifting the targeted position of the deletion cassette.

We confirmed successful deletion of each individual ORF by RFLP analysis. The inserted deletion cassette contained DraIII, HindIII, PvuI, NruI, and XhoI sites (Fig. 2). Therefore, cleavage with one of these restriction enzymes resulted in a change of the restriction pattern of the mutated BAC in comparison to the parental pBRFseR (see Fig. S2 in the supplemental material [http://www.vetmed.fu-berlin.de/en/einrichtungen/institute/we05/cowpox]), confirming the presence of the marker sequence. All mutants were checked with at least two different restriction enzymes (see Table S3 in the supplemental material). In all cases, the observed restriction pattern and the sequencing results were in complete agreement with the *in silico* predictions (data not shown).

Virus reconstitution. We performed virus reconstitution by transfecting Vero cells with BAC DNA in the presence of FWPV as helper virus. The obtained virus clones were passaged three times on Vero cells to remove FWPV helper virus. Of the 183 single ORF deletion mutants, 109 knockout viruses could be reconstituted, whereas reconstitution was unsuccessful for 74 knockout viruses (Table 1).

In order to minimize host range limitations of the reconstitution system, we used two independent systems to generate BAC-derived CPXV mutants. Mutants that did not reconstitute on

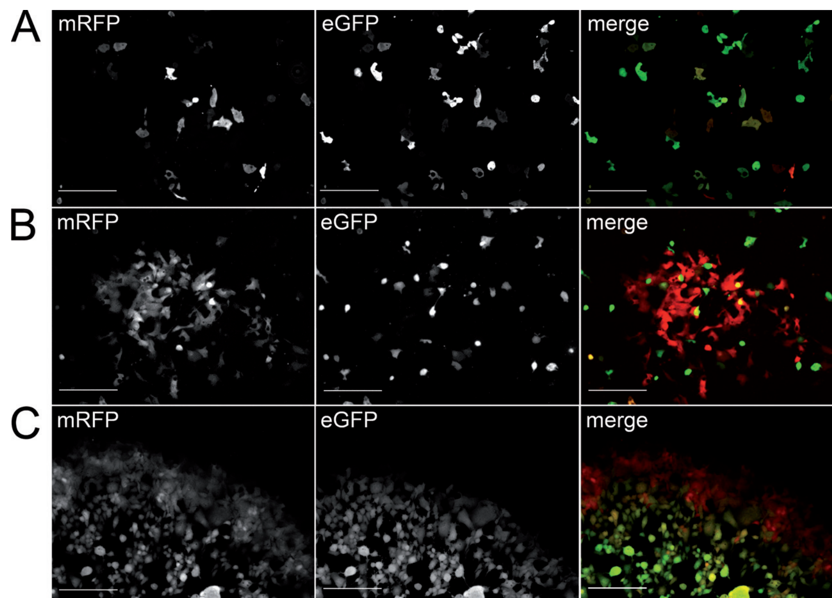

FIG 4 Different phenotypes upon reconstitution. (A) No plaque formation (vBRFseR-d195); (B) formation of small, "red fluorescence-only" plaques with single, double-fluorescent cells in the center (vBRFseR-d121); (C) formation of wild-type-like plaques that exhibit mRFP and eGFP fluorescence (vBRFseR-d158). Scale bars, 200 μm.

Vero cells in two independent experiments were also tested on CEC and SFV as a helper virus to confirm the result (see Materials and Methods). We termed genes "essential" if virus reconstitution failed twice with each of the two systems. Since it is well known that for VACV the *CPXV069* homolog *E3L* confers a host range phenotype on Vero cells (29, 30), we used BHK21 cells and SFV as the helper virus for reconstitution. Similar host range limitations might be true for other CPXV mutants that could not be reconstituted using the Vero and CEC systems and will be addressed in future studies.

Different phenotypes upon virus reconstitution. Signals emitted by mRFP and eGFP indicate early and late viral gene expression, respectively. Monitoring mRFP and eGFP fluorescence upon virus reconstitution or infection provided information about the involvement of individual CPXV genes in virus replication. According to the observed fluorescence signals, three different mutant virus phenotypes were observed: (i) no plaque formation, (ii) formation of small, red fluorescence-only plaques with single double-fluorescent cells in the center, and (iii) formation of wild-type-like plaques expressing both fluorescence markers (Fig. 4).

The red plaques expressed only mRFP but not eGFP except for a single cell in the center of the plaque (Fig. 4). The most likely explanation is that the FWPV helper virus was able to compensate for the deleted CPXV-BR gene in transfected cells. Due to complementation, the viral replication cycle could be completed in the central cell of the plaque, leading to the production of infectious viral particles. However, in neighboring cells that were infected subsequently with the mutant CPXV but not with helper FWPV, the replication cycle was aborted before the transition to late gene expression, as reflected by the lack of the green fluorescent marker. This hypothesis is supported by the fact that virus progeny was lost by repeated passage of progeny virus, which diluted and ultimately eliminated the helper virus.

Essential genes of CPXV. Wild-type-like formation of plaques by mutant viruses indicated that the gene product of the deleted ORF was dispensable for virus replication on Vero cells. This was the case for 109 of the 183 (60%) mutant viruses that we generated (Table 1 and Fig. 5). All six kelch-like protein single deletion mutants, as well as the mutant virus lacking all six genes, were also successfully reconstituted. During continuous passage on Vero cells, some mutant viruses exhibiting red and green fluorescent plaques lost their ability to produce infectious progeny. This suggested that the respective ORFs are essential for virus replication on these cells. The "red fluorescence-only" mutants (12/183) lost the ability to form plaques by repeated passage, and the respective mutants were consequently grouped with the mutants, for which no plaque formation was detectable from the start (62/183). Hence, the respective ORFs were considered essential for CPXV replication on Vero cells, and a total of 40% of the generated mutant viruses were grouped in this category.

Based on data from our mutant library, as well as information from the PBRC and previous studies performed on VACV (2, 4), a genome map was created to provide an overview about CPXV genes and their transcription kinetics (early, intermediate, and late) (Fig. 5). In accordance with previous studies, we found that 71% of all late genes were essential for virus replication. The products of all essential late genes are predicted to be incorporated into mature virions (24–26, 31). We also confirmed that most essential genes were located in the central portion of the genome, whereas most nonessential genes clustered toward the genomic termini. All except for 3 of the 74 genes determined to be essential were located in the central part of the CPXV-BR genome, where the central part accounts for 50% of the genome (Fig. 6). More than 50% (7/12) of the ORFs whose deletion resulted in the "red fluo-

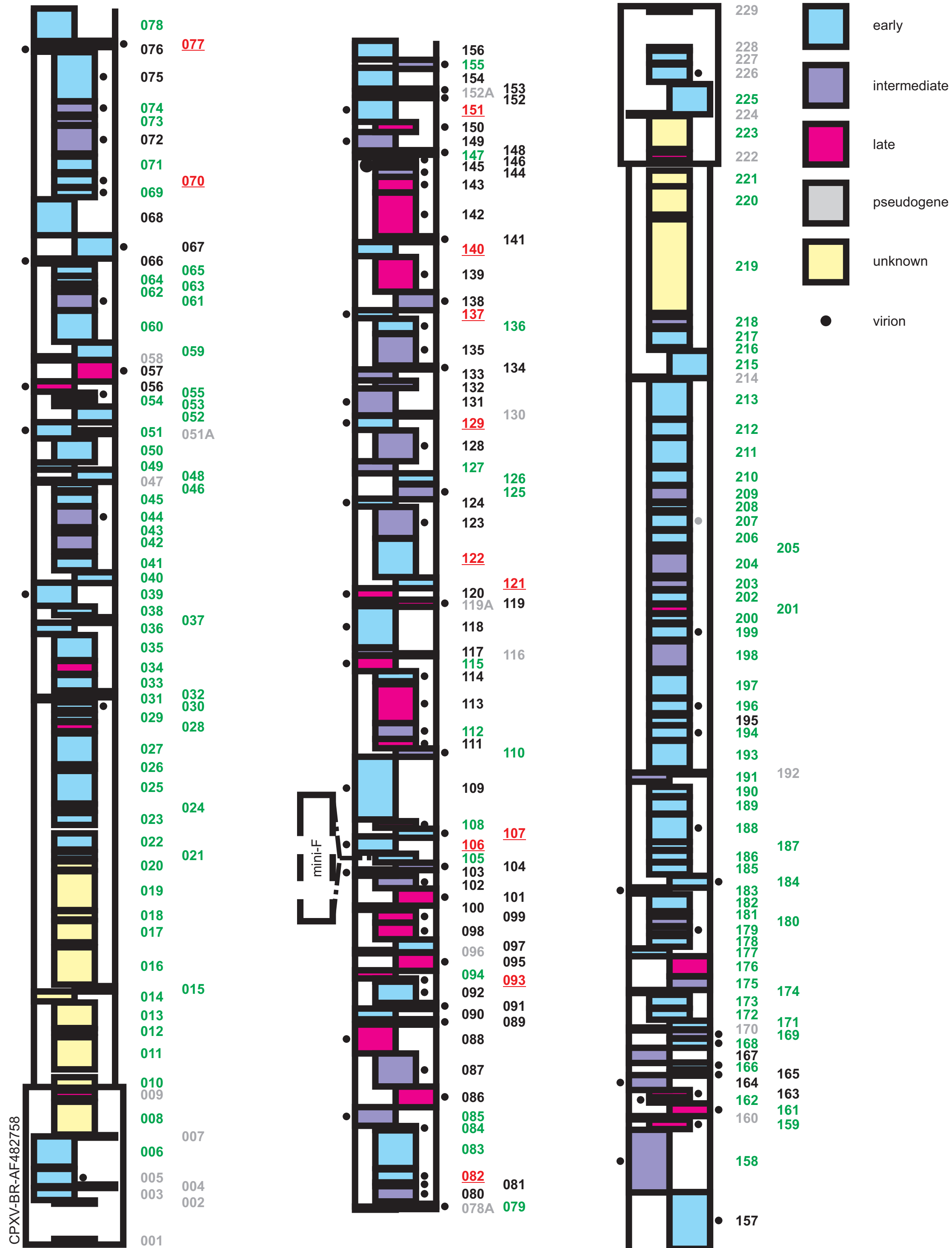

FIG 5 Overview of the results from reconstitution experiments of mutant BAC clones on Vero cells and CEC. The numbers of genes that are not essential for virus replication are highlighted in green. Deletion of "red" ORFs ("red fluorescence only"; red underline) resulted in virus progeny with a blocked transition to late gene expression. Gene numbers highlighted in black are essential, as characterized by the absence of production of any virus progeny upon three attempts at reconstitution. Gray numbers indicate putative ORFs, which were not deleted for generation of the library, since this group contains pseudogenes, small ORFs that completely overlap with other ORFs or genes within the TIR. The color of the ORFs indicates gene expression kinetics according to their homologues in VACV, as indicated on the right.

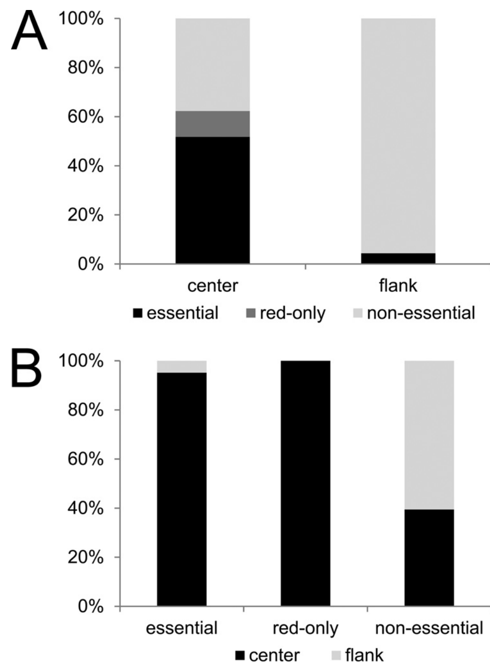

FIG 6 Distribution of essential and nonessential genes in the CPXV genome. (A) The flanking 50% of the CPXV genome consists almost exclusively of nonessential genes. (B) Most essential genes, as well as all genes of the "red fluorescence-only" phenotype, are located in the central 50% of the genome. However, the central part does also contain nonessential genes (~40% of the center genes are essential).

rescence-only" phenotype code for proteins that are involved in RNA transcription and are also predicted as structural components of the mature virion. Consistent with their essential nature, all 12 "red fluorescence-only" ORFs were located in the central part of the genome.

A previous extensive comparison of 21 completely sequenced poxvirus genomes revealed that 49 gene families are conserved among poxviruses and that 41 additional families are conserved in the *Chordopoxvirinae* (32). Based on this high degree of conservation, the authors of one study predicted these gene families to be essential for poxvirus replication (30), and some of the genes were already found to be essential by independent studies (Table 2). However, an exhaustive investigation to confirm this analysis of genes essential for poxvirus replication was lacking. We found that most of the conserved genes were also essential as derived from our data set, with the exception of 17 genes (Table 2), most of which are conserved in the *Chordopoxvirinae* but not all *Poxviridae*. Among these 17 genes, 10 have already been described to be nonessential in *in vitro* experiments (for references, see Table 2). For example, the VACV type I topoisomerase gene (H6R) is conserved among all poxviruses but is not essential for virus replication *in vitro* (33). However, the mutant virus exhibited reduced infectivity, which could be ascribed mainly to lower early transcription rather than to direct effects on the processing of viral DNA. Similarly, the VACV homologues D9R and D10R, which are encoding proteins likely involved in decapping host cell mRNAs, are not essential for virus replication if knocked out separately (34). A double deletion mutant for both ORFs failed, however, to reconstitute, possibly indicating mutually compensation of the two genes, which is most likely also the case in our knockout library. Clearly, further experiments are needed to confirm this hypothesis. Interestingly, we found *CPXV077* and *CPXV195* (homologs of VACV E11L and A57L, respectively) to be essential for virus replication *in vitro*, even though both are not conserved in poxviruses or chordopoxviruses.

In conclusion, we have created the first complete targeted BAC knockout library of a large DNA virus. Reconstitution of mutant clones has yielded novel insight into the importance of single viral genes for viral replication. With the insertion of fluorescent markers for early and late viral gene expression, the library at hand can be used for high-throughput screens to identify genes involved in

TABLE 2 Literature summary of nonessential conserved CPXV genes

CPXV-BR gene	VACV-COP homolog	Family name	Conservation[a]	Essential according to this study	Essential according to other publications	Reference
CPXV060	F12L	Actin tail, microtubule	P	No	No	34
CPXV061	F13L	Phospholipase extracellular enveloped virion	P	No	No	35
CPXV064	F15L	Unknown	P	No		
CPXV068	E2L	Unknown	P	No	No	36
CPXV074	E8R	Endoplasmic reticulum-localized MP	P	No		
CPXV084	I5L	Unknown VP13	P	No	No	37
CPXV085	I6L	Unknown	P	No		
CPXV108	J5L	Late MP, essential	C	No	Yes	38
CPXV110	H1L	Tyrosine-serine phosphatase	P	No	Yes	39
CPXV112	H3L	Intracellular mature virus morphogenesis viral protein (VP55)	C	No	No	40
CPXV115	H6R	Topoisomerase type I	C	No	No	32
CPXV126	D9R	mutT motif, nucleoside triphosphate pyrophosphohydrolase	P	No	No	33
CPXV127	D10R	Nucleophosphohydrolase-pyrophosphohydrolase downregulator	C	No	No	33
CPXV136	A4L	Core protein	P	No	Yes	41
CPXV147	A14.5L	IMV MP, virulence factor	P	No	No	42
CPXV155	A22R	Holliday junction resolvase	C	No	Yes	43
CPXV169	A34R	Extracellular enveloped virion glycoprotein	P	No	No	44

[a] According to a previously published study (28). C, gene families conserved in chordopoxviruses; P, gene families conserved in poxviruses.

various processes of the virus replication cycle, including features of host range, virulence factors, and gene products involved in immunomodulation.

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