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Generation of a Complete Single-Gene Knockout Bacterial Artificial Chromosome Library of Cowpox Virus and Identification of Genes involved in hemorrhagic lesions on infected chicken chorioallantoic membranes (CAMs)

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List of Abbreviations

BAC	Bacterial artificial chromosome
BSA	Bovine serum albumin
CAM	chicken chorioallantoic membranes
CEC	Chicken embryonic cell
CEV	cell-associated enveloped viruses
CPXV	Cowpox virus
Crm	cytoine response modifier
DNA	Deoxyribonucleic acid
dpi	Days post infection
E.coli	*Escherichia coli*
ECTV	ectromelia virus
EEV	extracellular enveloped virus
eGFP	Green fluorescent protein
FCS	Fetal calf serum
FWPV	fowlpox virus
GFP	green fluorescent protein
IC	immature virus
IEV	intracellular enveloped virus
IL	interleukin
IMV	intracellular mature virus
ITRs	inverted terminal repetitions
kb	kilo base pairs
MEM	Minimal essential medium
mini-F	Minimal fertility factor
MOCV	molluscum contagiosum virus
MPXV	monkeypox virus
mRFP	Red fluorescent protein
mRNA	Messenger RNA
MVA	Modified Vaccinia Ankara
OPV	orthopoxvirus
ORF	Open Reading Frame
pfu	Plaque forming unit
PKR	protein kinase R
RFLP	Restriction fragment length polymorphism
RFP	green fluorescent protein
RNA	Ribonucleic acid
RPXV	rabbit poxvirus
VACV	Vaccinia virus
VARV	Variola virus

Chapter 1: Introduction

1.1. Taxonomy of Poxviruses

Poxviruses are a family of large complex viruses that infect a broad variety of animal hosts including insects, birds, reptiles and mammals. Classification of poxvirus species is based on different criteria including natural host range, virulence, phylogenetic analysis, nucleotide sequence identity, and genome organization (1). According to their natural hosts, poxviruses are divided into two subfamilies, *Entomopoxvirinae* (poxviruses infect insects) and *chordopoxvirinae* (poxviruses infect vertebrates) (1). Taxonomical classification of the genera of the *Poxviridae*, the type species and their genome sizes are summarized in Table 1.1 (GenBank, www.ictvonline.org, www.poxvirus.org). Most of the poxviruses causing human diseases belong to the genus *Orthopoxvirus* (2).

Table 1. 1 Classification of *Poxviridae*

Subfamily	Genus	Type species	Genome Size of type specie	GenBank Accession number
Chordopoxvirinae	*Avipoxvirus*	*Fowlpox virus*	288,539 bp	NC_002188
	Capripoxvirus	*Sheeppox virus*	149,955 bp	AY077832
	Cervidpoxvirus	*Deerpox virus*	166,259 bp	NC_006966
	Leporipoxvirus	*Myxoma virus*	161496 bp	JX565563
	Molluscipoxvirus	*Molluscum contagiosum virus*	190289 bp	NC_001731
	Orthopoxvirus	*Vaccinia virus*	194,711 bp	NC_006998
	Parapoxvirus	*Orf virus*	139,962 bp	NC_005336
	Suipoxvirus	*Swinepox virus*	146,454 bp	NC_003389
	Yatapoxvirus	*Yaba monkey tumor virus*	134,721 bp	NC_005179
Entomopoxvirinae	*Alphaentomopoxvirus*	*Melolontha melolontha entomopoxvirus*	nn	-
	Betaentomopoxvirus	*Amsacta mooreientomopoxvirus 'L'*	232392	NC_002520
	Gammaentomopoxvirus	*Chironomus luridus entomopoxvirus*	nn	-

1.2. Orthopoxviruses

Among all poxviruses, orthopoxviruses (OPVs) have been best known and most intensively studied because some OPVs cause serious human diseases (1). Species in this genus are *Camelpox virus*, *Cowpox virus*, *Ectomelia virus*, *Monkeypox virus*, *Raccoonpox virus*, *Taterapox virus*, *Vaccinia virus*, and *Variola virus* (1). The most prominent OPV is the variola virus (VARV), which caused smallpox in human (3). Smallpox is a fatal human disease which killed millions of people globally (3). The terrible disease was eradicated worldwide in 1980 due to the effort of the World Health Organization and the effective smallpox vaccinaton with vaccinia virus (VACV) (3). Despite the eradication of smallpox, VARV is still considered as a bioterrorist threat and efforts are continuously made to develop more efficacious vaccines and new antivirals (4, 5). Since VACV is the vaccine for VARV, it is also the best characterized

poxviruses and most of the knowledge about poxvirus biology is derived from studies with VACV (6, 7, 8, 9, 10). Beside its use as smallpox vaccine VACV is also a suitable vector for developing recombinant vaccines (11, 12, 13).

1.3. Cowpox virus

Another important member of *Orthopoxvirus* genus is cowpox virus (CPXV). whose natural reservoirs are rodents (1, 14, 15). CPXV can infect a broad range of animals including cats, monkeys and elephants (16, 17, 18, 19). CPXV also causes zoonotic infections in Western Eurasia (20). Among all known OPVs, CPXV contains the largest and likely the most complete genome which contains all the genes found in other OPVs (15, 21, 22, 23). Although most studies investigating the function of OPV genes have been conducted using VACV, many of genes present in some OPV are absent from the VACV genome due to loss or truncation during the evolution of different species of the genus (24). Therefore, CPXV has become a popular model to study poxvirus biology and pathogenesis. A thorough investigation of CPXV biology may indeed lead to a better understanding of orthopoxvirus biology in general and ultimately provide information to develop a new class of safer vaccines.

1.4. Lesions caused by OPVs on chicken CAMs

All the OPVs cause distinctive visible lesions (pocks) on chorioallantoic membranes (CAM) of 10 to 12 days old chicken embryos. The studies of those pocks can date back before the advent of cell culture, when infections of CAMs were used to grow and characterize poxviruses (25, 26). Although all OPVs can infect chicken embryos and cause pocks on CAM, only CPXV and RPXV can cause hemorrhagic red pocks , whereas other OPVs produce nonhemorrhagic white pocks (27).

In early studies, spontaneous white pocks were observed during CAM infection of CPXV (28). However, CPXV proteins responsible for the white variant remained unknown. Later, CPXV protein CrmA (cytokine response modifier A) was proved to be involved in the hemorrhagic phenotype (29, 30). The CrmA belongs to the superfamily of serine protease inhibitors (serpin), which are found in plants, animals and bacteria (31). However, poxviruses are the only viruses known to encode functional serpins (31). Although CrmA was proved to inhibit the serine proteinase, granzyme-B and is a potent inhibitor of caspase-1 the role of CrmA in inducing hemorrhagic pocks on CAM is poorly understood (29, 32). In a previous study, the replacement of CrmA by myxoma virus serpin 2 or baculovirus P35 in CPXV restored the inhibition of the terminal caspase and the anti-apoptotic effects but not restored the induction of red pocks (33). Moreover, CPXV-BR produced white pocks when the serpin function of CrmA was abolished by a point mutation (33). In RPXV, products of serpin 1, serpin 2 (homolog of *crmA*) and ps/hr gene are responsible for the induction red-pock, while in CPXV, CrmA is the only known factor involved in the formation of red pocks (34).In a recent study, CPXV CrmA gene was introduced into genome of MVA. However, the expression of CrmA

failed to make the recombinant MVA to produce hemorrhagic pocks on chicken CAMs (35). This result suggests that CrmA is necessary but not sufficient for the red pock phenotype.

Besides CrmA, *kelch*-like proteins are considered to be potentially involved in the red pock phenotype (36). Kelch proteins are a superfamily of proteins diverse cellular functions and are found in various species including insects, mammals and viruses (37). Among all the OPVs, CPXV encodes six *kelch*-like proteins, VACV encodes three *kelch*-like proteins, MPXV encodes only one *kelch*-like protein, and VARV does not encode any functional *kelch*-like at all (38, 39). Considering the fact that OPVs encoding fewer *kelch*-like proteins produce white pocks and CPXV GRI-90 lacking four of the six *kelch*-like proteins produced smaller and whitish pocks, *kelch*-like might be involved in inducing red pocks on CAM (36). However, the function of orthopoxviral *kelch*-like proteins is poorly understood (38, 39).

1.5. Replication cycle, morphogenesis, and actin tail induction

Poxviruses are large complex DNA viruses that replicate entirely in the host cell cytoplasm (40, 41). Unlike most other viruses that have the symmetry structures such as helical or icosahedral, poxvirus virions appear as "brick shaped" particles with complex internal structures, consisting of a dumbbell-shaped core and lateral bodies (40, 41). The poxvirus virions are large compared to most other animal viruses. In the example of vaccinia virus (VACV), the virion has dimensions of approximately 360 x 270 x 250 nm (42).

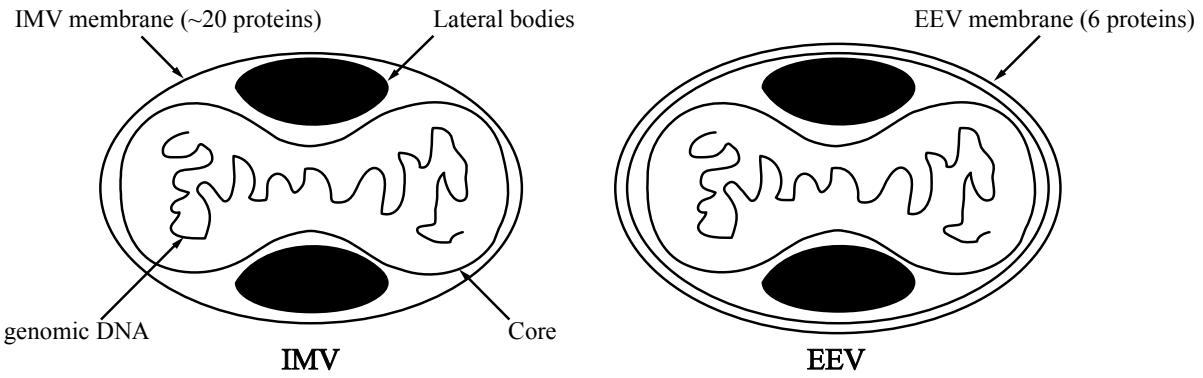

Figure 1. 1. Illustration of the morphology of poxviruses.
The two different infectious poxvirus particles are shown, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). Adopted from references (10), (43) and (40).

Two different forms of infectious virus particles are produced during infection, the intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Figure 1. 1) (44). IMVs are enveloped by one membrane that harbors approximately 20 proteins. EEVs are wrapped in an additional membrane that contains 6 proteins that are distinct from those in the IMV membrane (43, 45). IMVs are thought to mediate transmission between hosts, whereas EEVs mediate virus spread within the infected hosts (43, 45).

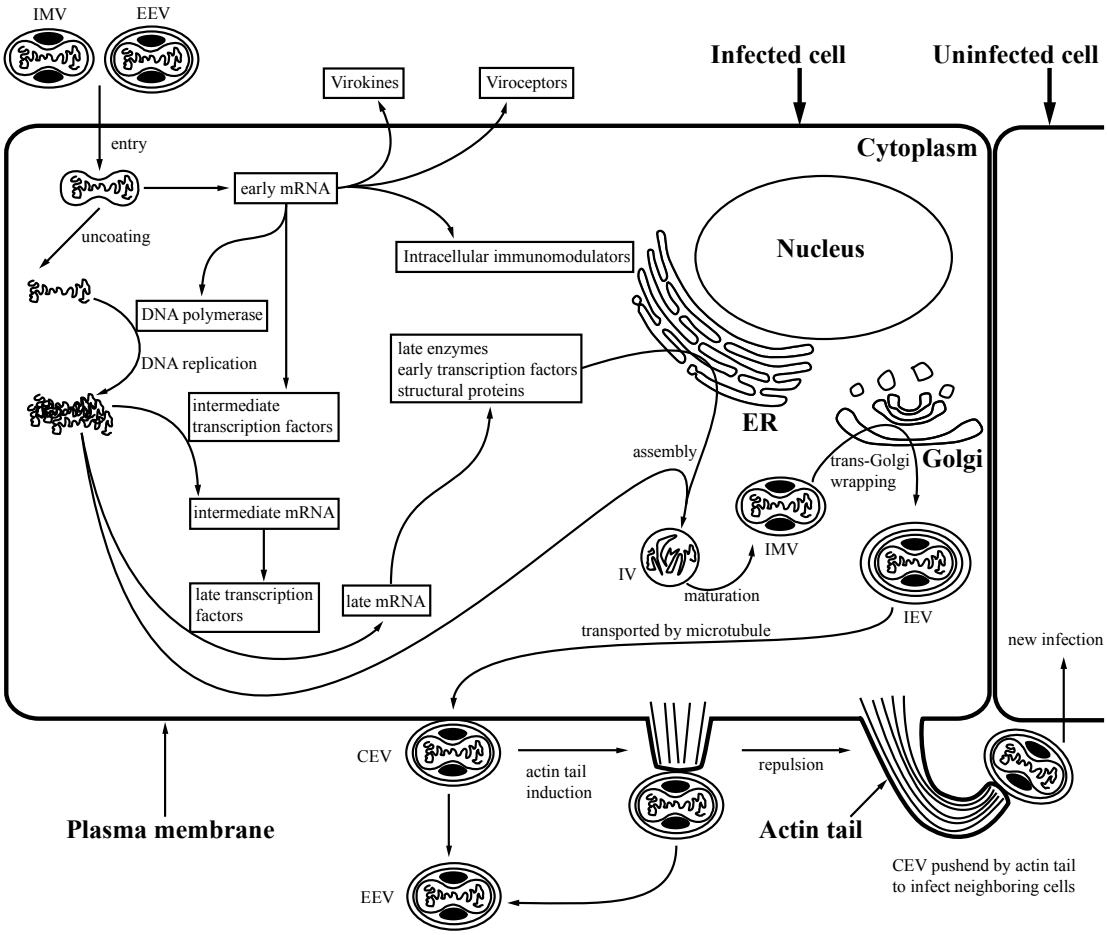

Figure 1. 2. Replication cycle, morphogenesis, and actin tail induction of poxviruses. Adopted from references (10), (46), (47), (7) and (44).

The replication cycle of poxviruses occurs entirely in the cytoplasm and is summarized in Figure 1 .2. The replication of poxviruses begins with the entry of virion into the cell through endocytosis or plasma membrane fusion, which requires ~11 viral proteins (43, 45). As the EEV has an additional membrane, the outer membrane of EEV is dissolved through interactions of cellular molecules and viral proteins (48). The viral core is released upon entry. The viral core already contains the complete early transcription system. Early genes are expressed to produce proteins that modulate host immune response, that are required for viral DNA replication and that are needed to start intermediate mRNA transcription. After the replication of viral genomic DNA, late gene expression is induced to generate mainly structural virus proteins (10, 44).

Virus morphogenesis is a complex process that follows DNA replication and late gene expression. During the morphogenesis of VACV, the first visible structure is half-moon shaped membranes, which can be observed 6 hpi and are termed as crescents (49). Crescents are derived from endoplasmic reticulum (ER) and grow into spherical structure called immature virus (IV). Then the viral genomic DNA is packaged into the IV and then IMVs are formed (40, 49, 50, 51, 52). The IMVs represent the majority of infectious progeny and is the basic infectious form of poxviruses. IMVs are released only upon cell lysis (41, 44). However,

some IMV particles are wrapped by two additional membranes derived from the Golgi and form intracellular enveloped virus (IEV). IEVs move to the cell surface, fuse with the plasma membrane and finally form EEV (41, 44). The transportation of viral particles with in the cytoplasm is mediated by microtubules. However, after the virus is transported to the plasma membrane, the actin cytoskeleton is induced to form actin tails. The actin tails repel EEV toward uninfected cells to establish new infection. This process of actin tails formation is already established upon early gene expression and also repulses newly attaching virions towards uninfected cells. In the way, the spread of viruses is greatly accelerated (7, 44).

1.6. Genome structure and DNA replication of poxvirus

Poxviruses contain large linear double strand genomes (130 kb-360 kb) and usually encoding more than 150 genes per genome (53). At each ends of the poxvirus genome are inverted terminal repeats (ITRs), which consist of identical but oppositely oriented sequences (54). Although hypervariable in length (55), the ITRs contain highly conserved regions of about 100 bp which forms a hairpin loop at the terminal that is required for the resolution of replicating DNA (56, 57). The hairpin loops are rich in A and T, and closes the ends of the two strands of the genomic DNA (58).

Poxvirus DNA replicates entirely in the cytoplasm of infected cells (59). Replication of viral DNA and assembly of mature virions form discrete cytoplasmic structures termed as viral factories containing proteins required for viral replication (60). In the example of VACV, DNA replication begins 1 to 2 hours after entering the cell and generates about 10,000 genome copies per cell, of which half are ultimately packaged into progeny virions (61, 62).

The mechanism of poxvirus DNA replication is not completely known. The popular replication model involves hairpin nicking and strand displacement (10). In this model, DNA replication begins with the introduction of a nick within one or both ITRs. The nick provides a primer for DNA synthesis in 5' to 3' direction (63). Small DNA fragments covalently linked to RNA serve as Okazaki fragments in the lagging strand synthesis (64).

DNA replication forms transient head-to-head and tail-to-tail genome concatemers that are resolved to produce single copy of genome DNA and form covalently closed hairpin ends in ITRs (58, 65). Poxvirus DNA concatemers accumulate when viral late protein synthesis was blocked and that head to tail concatemers appear as a result of recombination (66, 67). The head to tail configuration make it possible to circularize the genome DNA for generation of bacterial artificial chromosome (BAC) clone of the viral genome (68).

1.7. Regulation of Gene expression

Most studies about poxvirus gene expression were performed on VACV. According to their transcription at different stages during poxvirus replication, poxvirus genes can be classified as early, intermediate and late genes. Early genes are expressed immediately after infection,

intermediate and late genes are successively activated upon DNA replication (10). About half of VACV genes are transcribed early (69). Early transcription is based on the fact that viral cores already contains the compete transcription system which enables the synthesis of viral early proteins immediately after entry of virus cores. Viral mRNA is detectable within 20 minutes and reaches a peak of synthesis at 100 minutes after infection. Products of early genes include enzymes required for DNA replication, new viral RNA polymerase and capping enzyme, host defense functions and intermediate gene transfection factors. Products of intermediate genes are enzymes and factors required for late gene expression. Products of late genes included the early transcription factors, which are packaged in progeny virions (70).

Promoters appear to be conserved among different poxviruses. They are also functional in a cell infected with a different poxvirus (71, 72). Early promoter contains a consensus A/T-rich sequence (AAAAAATGAAAAAA/TA) which locates 12-17 nucleotides upstream of the start site of RNA transcription (73). Intermediate promoters contain two important regions, a core element and an initiator element. The core element is a 14 bp A/T rich sequence that located 10 or 11 bp upstream of the transcriptional start site. The initiator has a sequence of TAAAT/A at nt -1 to +4 of the start site (74). Late promoters also contain two regions, a core structure and a highly conserved sequence at the initiation site. The core sequence contains about 20 nucleotides rich in A and T. The highly invariable sequence is TAAAT (75).

1.8. Modulation of host immune response by OPVs

To successfully replicate within their hosts, poxviruses encode multiple proteins to interfere host immune response. These viral proteins can be grouped into three categories, (i) virokines, which are secreted and resemble host regulatory molecules; (ii) viroceptors, which are secreted or surface localized proteins that bind and sequester immune ligands; (iii) intracellular modulators, which inhibit cellular signaling pathways (47, 76, 77).

(i)Virokines. Poxviruses can encode secreted proteins that mimic host regulatory molecules such as cytokines and complement regulators and act as competitive inhibitors. One virokine encoded by poxvirus is interleukin-18 (IL-18) binding protein. IL-18 is a proinflammatory cytokine that induces IFN-γ, regulates synthesis of various cytokines, controls Th1 and Th2 responses, and activates cytolytic effector cells (78). Ectromelia virus (ECTV), Molluscum contagiosum virus (MOCV), VACV and CPXV have been shown to encode functional viral IL-18 binding protein (IL-18BP) which blocks IL-18 (76, 77, 79, 80, 81).

(ii) Viroceptors. Poxviruses can also encode soluble viral receptors that capture host immune regulatory molecules to modulate host immune response. For example, poxviruses can block the activity of interferons (IFN) by encoding to proteins that are similar to IFN receptors to prevent IFN binding to its natural receptor. IFNs are a group of secreted cytokines that activate innate immune response to viral infection. For example, the B19 protein encoded by VACV Copenhagen (COP) strain was shown to competitively bind and block type I IFNs (82, 83), and the VACV B8 protein was proved to bind and inhibit IFN-γ (84).

(iii) Intracellular modulators. Excepts virokines and viroceptors, poxviruses also encode intracellular proteins that modulate various proinflammatory signaling pathways. The dsRNA-dependent kinase R (PKR) initiates cascades that inhibit viral protein synthesis and is activated by double-stranded RNA (dsRNA) produced during poxviral transcription (85). E3 protein expressed by VACV was proven to inhibit the activity of PKR (86). Other poxviruses such as CPXV, ECTV and MOCV also encode homologs of E3 (76, 77). Poxviruses also encode other intracellular modulators. The CPXV CrmA is a serpin that inhibits granzyme B and caspase-1 and is involved in the modulation of inflammatory cytokines (87, 88, 89).

1.9. Outline of this thesis

This thesis consists two parts. In the first part, a knockout library of the genome of CPXV strain Brighton Red was constructed, based on a previously described full-length BAC clone termed pBRF (92). Mediated by Red recombination in *E. coli* single single open reading frames (ORF) of CPXV BR were deleted by the insertion of a sequence containing stop codons and a kanamycin resistance gene. Upon reconstitution of each of the mutant virus genomes, different viral phenotypes were characterized and genes essential or non-essential for CPXV replication were identified.

In the second part, single deletion viruses reconstituted in the first part and kelch-like deletion mutants were used to screen for factors involved in the red pock phenotype on CAM. Most of the mutant viruses produce hemorrhagic red pocks on infected CAM, while 10 single deletion mutants induced non-hemorrhagic white pocks.

Chapter 2 Generation of a Complete Single-Gene Knockout Bacterial Artificial Chromosome Library of Cowpox Virus and Identification of Its Essential Genes

Zhiyong Xu, Dimitrios Zikos, Nikolaus Osterrieder and B. Karsten Tischer

2.1. Abstract

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 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.

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Chapter 3. Identification of 10 cowpox virus proteins that are necessary for induction of hemorrhagic lesions (red pocks) on chorioallantoic membranes

Zhiyong Xu, Dimitrios Zikos, Aistė Tamošiūnaitė, Robert Klopfleisch, Nikolaus Osterrieder and B. Karsten Tischer

3.1. Abstract

Cowpox viruses (CPXV) cause hemorrhagic lesions ("red pocks") on infected chorioallantoic membranes (CAM) of embryonated chicken eggs, while most other members of the genus *Orthopoxvirus* produce non-hemorrhagic lesions ("white pocks"). Cytokine response modifier A (CrmA) of CPXV strain Brighton Red (BR) is necessary but not sufficient for the induction of red pocks. To identify additional viral proteins involved in the induction of hemorrhagic lesions, a library of single gene CPXV knockout mutants was screened. We identified 10 proteins that are required for the formation of hemorrhagic lesions, which are encoded by *CPXV060*, *CPXV064*, *CPXV068*, *CPXV069*, *CPXV074*, *CPXV136*, *CPXV168*, *CPXV169*, *CPXV172* and *CPXV199*. The genes are the homologs of *F12L, F15L, E2L, E3L, E8R, A4L, A33R, A34R, A36R* and *B5R* of vaccinia virus (VACV). Mutants with deletions in *CPXV060*, *CPXV168*, *CPXV169*, *CPXV172* or *CPXV199* induced white pocks with a comet-like shape on the CAM. The homologues of these five genes in VACV encode proteins that are involved in the production of extracellular enveloped viruses (EEV) and the repulsion of superinfecting virions by actin tails. The homologue of CPXV068 in VACV is also involved in EEV production but not related to actin tail induction. The other genes encode immunomodulatory proteins (*CPXV069* and *crmA*) and viral core proteins (*CPXV074* and *CPXV136*), and the function of the product of *CPXV064* is unknown.

Buy it:

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Chapter 4. Discussion

4.1. Cowpox virus is a suitable model to study orthopoxviruses

CPXV belongs to the genus *Orthopoxvirus*, which includes VARV, an exclusive human pathogen, and other zoonotic pathogens, such as CPXV and MPXV (3, 15, 20). CPXV can be used as a suitable model for studying OPVs because of several reasons. Firstly, CPXV has the largest genome (about 220 kb) of all OPV, about 30 kbp larger than that of VACV. Moreover, CPXV possesses the most complete genome in terms of number of genes as well as some unique ORFs (22, 97). Therefore, CPXV was suggested to be the most closest to the common ancestor of OPVs (22). Secondly, 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 (20). This could lead us to find out OPV genes that are involved in the host range determination of OPVs. Finally, the cloning of the CPXV BR as a BAC enables us to efficiently and precisely modify the genome of CPXV (35, 92).

As most of the studies about OPVs were performed on VACV, which is mainly non virulent and lacks functional copies of several OPV genes (24), the study of CPXV will provide new information about OPVs. In this thesis, a knockout library of CPXV was generated based on a CPXV BAC clone. By screening the knockout library, new phenotypes were observed, essential and non-essential genes of CPXV were identified, and CPXV genes involved in the induction of hemorrhagic lesions on CAM were discovered. These findings are the first benefits of this knockout library, which might be used in more studies in the future.

4.2. Advantages of BAC technology in studying poxvirus

BACs represent a cloning system for large genomic DNA based on minimal F-factor plasmids (98, 99). Large DNA fragments up to 300 kb can be cloned into the F-factor vector and transformed into *E.coli* that maintain BAC DNA (98, 99). In the field of molecular virology, BAC technology is a powerful tool to propagate and modify large viral genome and has been used to clone genomes of several poxviruses (68, 92, 93, 94, 100). The BAC cloning system has been proved to stably to maintain poxvirus genomes. In the previous studies, it was shown, that integrity of poxvirus sequences is maintained even upon various targeted mutagenesis (93, 101). This result underlines the suitability of the BAC technology for cloning and modifying poxvirus genomes in *E.coli*.

Besides the stability of BAC clones, methods of modifying BAC DNA are also more advantageous than classical methods to modify poxviral genomes. Classical methods are based on homologous recombination in infected vertebrate cells (90). Because of low efficiency of homologous recombination, mutants containing the desired,

modified genome are rare among the virus progeny and multiple rounds of plaque selection, purification and sometimes even chemical selection or comprehensive screening protocols are needed (90). Deletion of genes that are important for virus replication is much more difficult due to lower fitness of mutant viruses that will result in poor replication rates or would require the use of complementing cell lines (91). Modification of viral BAC clones in prokaryotic cells avoids such problems. Recombination of cloned viral sequences can be accomplished without the selective pressure of virus fitness and multiple consecutive modifications can be done without the need individual purification steps (100). Efficient mutagenesis methods such as *en passant* mutagenesis mediated by Red recombination even allow markerless modifications of BAC DNA (95, 96).

A BAC clone (named as pBR) of CPXV Brighton Red strain was constructed previously by inserting the mini-F into the locus of the non-essential thymidine kinase (TK, *BR-105*) in our lab (92). Growth properties of virus reconstituted from pBR are similar to that of wild-type virus or a recombinant virus without the mini-F (92). Precise and deliberate modification of this BAC clone has been performed to study biological properties of CPXV in a previous study (35). By using the BAC technology and Red recombination methods, a single gene knockout BAC library was generated, which leads to the systemic study of CPXV.

4.3. The knockout library of CPXV is a novel tool to study OPVs

Although studies based on CPXV and VACV BACs were performed previously (35, 93), no systemic and complete knockout study was conducted on either CPXV or VACV.

The single gene knockout library of pBRFseR was generated by the Red recombination mediated introduction of a sequence containing in frame premature stop codons and a downstream bacterial selection marker (96). All the insertions were designed deliberately to avoid interruption of neighboring ORFs as well as promoters for adjacent ORFs. The in frame stop codons will precisely abrogate the expression of the targeted gene while the stably inserted bacterial selection marker protects from unwanted reversions upon virus reconstitution. In this study, 183 single ORF deletion BAC clones and a serial of *kelch*-like deletion mutant BAC clones were generated. Except the ankyrin repeat protein-encoding genes and pseudo genes, this knockout library covers all the single CPXV ORFs.

The knockout library is not only the first poxvirus knockout library, but also is the first complete targeted BAC knockout library of a large DNA virus. It can be used as a novel tool for the systematic characterization of OPV genes. The library allows high-throughput screeningas the knockout viruses encode both mRFP and eGFP, and the fluorescent signals can be monitored with multiwell readers. Similarly, CPXV

genes involved in immunomodulation can be screened by measuring production of cytokines in each well.

4.4. Identification of essential and non essential genes

Previous bioinformatic analysis of 21 completely sequenced poxvirus genomes identified 49 poxvirus gene families that are conserved in both the entomo- and chordopoxviruses, and 41 gene families that are conserved among chordopoxviruses (102). This information could possibly lead to definition of the minimum poxvirus genome and provide guidelines for future research. Because this analysis was based on experimental data, there remains a high degree of uncertainty. In contrast, our study is based on a complete library of deletion mutants of CPXV and as such provides “real virus” data. In this way, the complete single knockout library of CPXV could provide experimental data to verify the result of computational DNA analysis. Moreover, the identification of CPXV essential and non-essential genes could be valuable for studying OPVs.

In this study, mutant BAC clones that could not be reconstituted are essential genes for CPXV replication. The reconstitution of knockout mutant from BAC clones is more advantageous than direct knockout of genes. Classical methods of modifying poxvirus genome is laborious and time consuming due to low efficiency of homologous recombination, which make it difficult to create a complete set of single gene knockout mutants to screen the essential and non-essential genes. Additionally, deletion mutants of genes that are very important for virus replication are difficult to generate by homologous recombination. As a result, such genes will be classified as essential. However, deletion mutants reconstituted from BAC knockout mutants can avoid these problems. A disadvantage of the BAC knockout is that the viral reconstitution system may lead to the problem of host range limitation. But two different sets of cells and helper viruses were used in this thesis. In this way, the possibility of host range limitation was minimized.

Among the 74 knockout mutants that could not been reconstituted, 12 mutants produced red-only plaques upon reconstitution and failed to produce any plaque during passage. This red plaques are formed because of the complementation by the helper virus, which suggests that the corresponding genes can be complemented by homologs in helper virus. According to our data of virus reconstitution, all the essential genes belong to previously described conserved poxvirus gene families (102). However, 16 conserved genes are non-essential for replication of CPXV in cell culture, suggesting that these genes are conserved but not required for CPXV replication in Vero cells. It is also possible that homologs of these 16 conserved but non-essential genes are essential in some other poxviruses. In this regard, when studying essential and non-essential genes in other poxviruses, homologs of these 12 genes involved in the formation of red-only plaque and these 16 conserved should be

top candidates. Therefore, the complete targeted BAC knockout library of CPXV and the consequent identification of CPXV essential and non-essential genes have yielded novel insight into the importance of single genes for viral replication.

4.5. Identification of CPXV genes involved in red pock phenotype

Another application of the knockout library was the screening of CPXV proteins involved in inducing hemorrhagic lesions (pocks) on chorioallantoic membranes (CAM) of chicken embryos. All OPVs can infect chicken embryos and cause distinctive visible pocks on CAMs at 2 to 4 days post-infection (dpi). However, the only known OPVs that can produce hemorrhagic pocks are CPXV and RPXV (27). Although the CPXV CrmA (cytokine response modifier A) has been proved to be responsible for inducing the hemorrhagic pocks, it was shown that the protein is not sufficient for inducing the hemorrhagic phenotype (29, 35). However, other CPXV proteins involved in this phenotype remained unknown.

The CPXV knockout library enabled us to identify other genes that have an impact on this phenotype. In this thesis, all 109 single deletion mutants and *kelch*-like deletion mutants was screened by infecting chicken CAMs. The mutant vBRFseRd061 failed to produce any pocks, which suggests that the protein CPXV061 might be involved in host range determination. From all these single knockout mutants, 10 single deletion mutants produced white pocks on the chicken CAM. The results were verified by the generation of revertant mutants, which restored the red-pock phenotype. The newly identified 10 proteins involved in red pock formation are encoded by *CPXV060*, *CPXV064*, *CPXV068*, *CPXV069*, *CPXV074*, *CPXV136*, *CPXV168*, *CPXV169*, *CPXV172* and *CPXV199*. The homologous genes in vaccinia virus (VACV) are *F12L, F15L, E2L, E3L, E8R, A4L, A33R, A34R, A36R* and *B5R*.

All the 10 proteins are highly conserved among members of the genus *Orthopoxvirus* according to bioinformatic analysis (102). BLAST results also revealed a high degree of similarity of the 10 CPXV-BR proteins and their homologs in different OPVs. This analysis suggests that the functions of the identified proteins in CPXV are comparable to those of their counterparts in other OPVs. Therefore, functions of the 10 proteins are predicted according to studies on the respective protein in VACV and then divided into three different categories, immunosuppressive proteins, core proteins, and EEV related proteins. Earlier studies on the induction of hemorrhage upon CPXV infection of CAM only focused on modulators of host immune responses (33, 35). In this thesis, another two categories of structural proteins (core and EEV) are proved to be necessary for inducing hemorrhagic lesions. These findings provide novel insights into the possible mechanism of the hemorrhagic phenotype on chicken CAM of CPXV.

Interestingly, pocks produced by deletion mutants of *CPXV060, CPXV168, CPXV169,*

CPXV172 or *CPXV199* are comet-like which is similar to ps/hr gene (homologous to *CPXV199* and VACV *B5R*) deletion mutant of RPXV (27). Proteins encoded by the 5 proteins are involved in actin tail formation and/or EEV production (44). The deletion of actin-tail related genes may alter the way of virus transmission, which might have caused the different pock shape. As previous studies concerning actin tail repulsion were conducted in cell culture (7, 103), the CAM infection system will provide an optimal model to study the spread of poxvirus *in vivo*. The five mutants that produce comet-like pocks can be used in future research to investigate the role of actin induction in transmission of poxviruses.

As the CAM is a tissue that lacking lymphocytes and adaptive immune system (29, 30, 33), it is also a good model to study immune responses and immunomodulation of CPXV. Our results have shown that all the single gene knockout mutants except vBRFseRd61 and *kelch*-like deletion mutants are being able to infect the CAM and induce lesions, which make it possible to study the role of single CPXV gene in immunomodulation. High-throughput *in vitro* screening of CPXV mutants will provide information about immunomodulation of CPXV genes. In this way, the number of possible candidates for *in vivo* study can be narrowed down and make the study more efficient.

Summary

Generation of a Complete Single-Gene Knockout Bacterial Artificial Chromosome Library of Cowpox Virus and Identification of Genes involved in hemorrhagic lesions on infected chicken chorioallantoic membranes (CAMs)

Cowpox virus (CPXV) belongs to the genus *Orthopoxvirus* in the family *Poxviridae*. CPXV is a large complex virus that infects a broad range of animals and can cause zoonotic infections. CPXV has the largest genome and the most complete set of genes among the orthopoxviruses, therefore CPXV has become a popular model for studying poxvirus genetics and pathogenesis. The bacterial artificial chromosome (BAC) clone of CPXV Brighton Red (BR) strain constructed in a previous study has enabled us to efficiently modify the CPXV genome for studying the virus.

In this thesis, a complete knockout library of the BAC clone of CPXV BR was generated and characterized. These mutants allow high-throughput and systematic assessments of the roles of single CPXV genes in virus infection and replication, as well as virus host range determination. Out of 183 mutant BAC clone, 109 mutant viruses were successfully reconstituted, suggesting that the 109 corresponding genes are not necessary for virus replication while the other 74 ORFs are essential. As all mutant BAC clones contain dual fluorescent markers for early and late viral gene expression virus reconstitution can be monitored easily. Thus it was possible to discriminate between different phenotypes, such as no plaque formation, formation of 'red fluorescence-only' plaques, and formation of wild-type-like 'red and green fluorescence' plaques.

The knockout library was also applied for another systematic screening, the identification of CPXV proteins involved in inducing hemorrhagic lesions ("red pocks") on chicken chorioallantoic membranes (CAM). Although all orthopoxviruses can infect chicken CAM, only CPXV and rabbit poxvirus induce red pocks. Other orthopoxviruses produce non-hemorrhagic lesions ("white pocks"). Cytokine response modifier A (CrmA) of CPXV proved to be necessary but not sufficient for the induction of red pocks. Another study showed that *kelch*-like proteins might also be involved in the hemorrhagic phenotype. By screening all the single knockout mutants and *kelch*-like deletion mutants, 10 proteins that are required for the formation of hemorrhagic lesions were identified and *kelch*-like proteins were proved to be not responsible for inducing red pocks.

In conclusion, the first complete targeted BAC knockout library of a large DNA virus was generated in this thesis. Reconstitution of mutant BAC clones provided important information about the importance of single viral genes for viral replication, and led to the identification of new phenotypes of CPXV. By screening CAM infection of the reconstituted mutant viruses, 10 proteins that are involved in inducing hemorrhagic lesions on chicken CAM was identified. This discovery provided novel insights into the mechanisms of the formation of hemorrhagic lesions. In the future, this knockout library can be used for more high-throughput screens to study various biological characteristics of CPXV.

Zusammenfassung

Erstellung einer vollständigen Einzelgen-Knockout Bibliothek des Kuhpockenvirus und Identifizierung von Genen, die an der Bildung hämorrhagischer Läsionen auf chorioallantoischen Membranen infizierter Hühnereier beteiligt sind

Kuhpocken (CPXV) gehören zur Gattung der Orthopockenviren in der Familie *Poxviridae*. CPXV ist ein großes komplexes Virus, das in der Lage ist ein breites Speziesspektrum zu infizieren und Zoonosen zu verursachen. CPXV hat das größte und vollständigste Genom unter den Orthopockenviren und ist deshalb ein beliebtes Model zur Untersuchung der Genetik und Pathogenese von Pockenviren geworden. Ein BAC (bacterial artificial chromosome)-Klon von CPXV, der in einer früheren Studie erstellt wurde, ermöglichte die effiziente Modifikation des CPXV Genoms um das Virus zu studieren.

In der hier vorgestellten Arbeit haben wurde eine vollständige Einzelgen-Knockout Bibliothek auf Basis des CPXV-BAC-Klons erstellt und charakterisiert. Die resultierenden Mutanten ermöglichen systematische Hochdurchsatzuntersuchungen der Rolle einzelner CPXV-Gene bei viraler Infektion, Replikation und der Festlegung des Wirtspektrums. Von 183 Mutanten konnten 109 mutierte Viren erfolgreich rekonstituiert werden, was darauf hinweist, dass diese 109 Gene, im Gegensatz zu den verbleibenden 74 Genen, für die Virusreplikation nicht essentiell sind. Alle mutierten BAC-Klone enthielten zwei Fluoreszenzmarker für frühe und späte virale Genexpression, was es uns ermöglichte neue Rekonstitutionsphänotypen in Zellkultur zu beschreiben.

Darüber hinaus wurde die Bibliothek auch auf einen anderen Phänotyp hin systematisch untersucht, die Bildung von hämorrhagischen Läsionen („Rote Pocken“) auf chorioallantoischen Membranen von Hühnereiern (CAM). Obwohl alle Orthopockenviren Hühner-CAMs infizieren können, induzieren nur CPXV und Kaninchenpocken „Rote Pocken“, während alle anderen Orthopocken nur nicht-hämorrhagische Pocken („Weiße Pocken“) hervorrufen. Das CrmA-Gen („Cytokine response modifier A“) der Kuhpocken wurde als notwendig, aber nicht hinreichend für die Induktion von Roten Pocken bestätigt. Andere Studien haben gezeigt, dass die Kelch-ähnlichen Proteine möglicherweise an der Bildung dieses Phänotyps beteiligt sind. Durch einen Screen aller Mutanten sowie von Klech-Deletionsmutanten waren wir in der Lage 10 Proteine zu identifizieren, die für die Ausbildung hämorrhagischer Läsionen notwendig sind und konnten zeigen, dass Kelch-ähnliche Proteine nicht an der Bildung von Roten Pocken beteiligt sind.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 28.04.2014 Zhiyong Xu