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Generation of an Infectious Clone of Duck Enteritis Virus (DEV) – A Basis for

Pathogenesis Studies and Vectored Vaccine Development

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Abbreviations

aa	Amino acid
AC-ELISA	Antigen-capture enzyme-linked immunosorbent assay
AIV	Avian influenza virus
BAC	Bacterial artificial chromosome
BF	Bursa of Fabricius
BHV-1	Bovine herpesvirus type 1
BHV-4	Bovine herpesvirus type 4
BHV-5	Bovine herpesvirus type 5
bp	Base pair
BVDV	Bovine viral diarrhea virus
CEC	Chicken embryo cells
CIV	Canine influenza virus
CPE	Cytopathogenic effect
DEC	Duck embryo cells
DEV	Duck enteritis virus
DNA	Deoxyribonucleic acid
DP	Duck plague
dsDNA	Double-stranded DNA
DVE	Duck viral enteritis
EHV-1	Equine herpesvirus type 1
EHV-4	Equine herpesvirus type 4
EIV	Equine influenza virus
ELISA	Enzyme-linked immunosorbent assay
EMEM	Earle's minimal essential medium
FBS	Fetal bovine serum
FHV-1	Feline herpesvirus type 1
G+C	Guanine-cytosine content
GaHV-3	Gallid herpesvirus 3
gC	Glycoprotein C
GFP	Green fluorescent protein
GPCMV	Guinea pig cytomegalovirus
h	Hour

HA	Hemagglutinin
HCMV	Human cytomegalovirus
HHV-6A	Human herpes virus 6A
HPAI	Highly pathogenic avian influenza
HSV-1	Herpes simplex virus type 1
HVT	Herpesvirus of turkeys
IBD	Infectious bursal disease
ICS	Immunochromatographic strip
IIF	Indirect immunofluorescence
ILTV	Infectious laryngotracheitis virus
IRS	Internal repeat short
kDa	Kilo Dalton
kbp	Kilo base pairs
KSHV	Kaposi's sarcoma-associated herpesvirus
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani
MCMV	Murine cytomegalovirus
MDV	Marek's disease virus
MHV-68	Murine gammaherpesvirus 68
MOI	Multiplicity of infection
NA	Neuraminidase
nm	Nanometer
NT	Neutralization tests
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
P_{HCMV}	Promoter of human cytomegalovirus
p.i.	Post-infection
PRV	Pseudorabies virus
qRT-PCR	Quantitative real-time PCR
RFLP	Restriction fragment length polymorphism
rhLCV	Rhesus macaque lymphocryptovirus
RNA	Ribonucleic acid

RPHA	Reverse passive hemagglutination
SDS	Sodium dodecyl sulfate
ssDNA	Single-stranded DNA
SVV	Simian varicella virus
TCID$_{50}$	Tissue culture infectious doses 50
TG	Trigeminal ganglia
TRS	Terminal repeat short
UL	Unique long
US	Unique short
VZV	Varicella-zoster virus

Zusammenfassung

Generierung eines infektiösen Duck Enteritis Virus (DEV) Klons
– Eine Basis für Pathogenitätsstudien und Vektor basierende Impfstoffe

Diese Arbeit beschäftigte sich mit der Konstruktion eines infektiösen *Bacterial Artificial Chromosome* (BAC)-Klons des Duck Enteritis Virus (DEV) Stammes 2085. Dieser infektiöse Klon ermöglichte die Generierung eines Vektor-basierten DEV-Impfstoffes, welcher das Hämagglutinin (H5) eines hochpathogenen H5N1 aviären Influenzavirus (AIV) exprimiert. Im Anschluß wurde die gesamte Genomsequenz des DEV-Stammes 2085 bestimmt und mit dem Genom der DEV-Stämme VAC, Clone-03 und CHv verglichen. Für die Konstruktion des infektiösen Klons wurde eine Mini-F-Vektorsequenz mittels homologer Rekombination an Stelle des UL44 (gC) Gens in das Genom des Stammes 2085 eingebracht. Die DNA des daraus resultierenden rekombinanten Virus wurde in den *Escherichia coli*-Stamm MegaX eingebracht und daraus resultierende Klone analysiert. Derr korrekte DEV-BAC-Klon (p2085) wurde für spätere Mutagenese in den *E. coli*-Stamm GS1783 elektroporiert. Die Transfektion von Hühner-Embryofibroblasten mit p2085 führte zu DEV-spezifischen Plaques, die nach UV-Anregung grüne Autofluoreszenz aufwiesen, Dies ließ auf eine erfolgreiche Generierung eines infektiösen Klons des DEV Stammes 2085 schließen. Eine gC-negative Mutante wurde durch Deletion der Mini-F-Sequenzen mithilfe des Cre-Lox Rekombination genriert. Zudem wurde eine Virus-Revertante durch Ko-Transfektion von p2085 mit einem PCR-Produkt, das UL44-Sequenzen enthielt, erzeugt. Schließlich wurden H5-Sequenzen in p2085 eingebracht, um ein rekombinantes Virus herzustellen, welches das Hauptimmunogen des AIV exprimiert. Eine robuste Expression von H5 in mit der Rekombinante infizierten Zellen wurde durch indirekte Immunfluoreszenz sowie Western Blot nachgewiesen. Plaque-Größenbestimmungen zeigten, dass die Plaque-Größen von der gC-negativen Mutante signifikant größer (12%) waren als die des Parentalvirus 2085 oder der Revertanten (ANOVA, $P<0,05$), während die Plaquegrößen von der Fremdgene (H5 oder GFP) exprimierten, signifikant reduziert waren. Zwischen den DEV-Mutanten und parentalem oder revertantem DEV wurden keine signifikanten Unterschiede in den intrazellulär vorhandenen Virustitern bestimmt. Dagegen ließen Virustiter der Überstände infizierter Zellen eine signifikante Abnahme um mehr als das 700-fache im Falle des gC-negativen Viruses im Vergleich mit parentalem 2085 Virus erkennen. Zell-assoziierte Virustiter des gC-negativen DEV zeigten ebenfalls eine signifikante Abnahme um das 50- bis 500-fache gegenüber dem Elternvirus (ANOVA, $P<0,05$).

Des Weiteren wurde das Genom der 2085 Stammes anhand des generierten BAC sequenziert. Das 2085-Genom ist 160.600 bp lang und kodiert für 78 offene Leserahmen (ORFs) (GenBank ID: 999965), eine Anzahl, die mit der des attenuierten DEV VAC-Stammes identisch ist (GenBank ID: EU082088.2). Ein Vergleich der Genomsequenzen von DEV 2085 und VAC mit Teil-Sequenzen des virulenten CHv Stammes und dem attenuierten Stamm Clone-03 wurde durchgeführt, um Nukleotid- oder Aminosäuren-Polymorphismen zu identifizieren, die potentiell zur Virulenz von DEV beitragen. In 24 der 78 ORFs wurden keine Aminosäure-Veränderungen identifiziert. Ein Ergebnis, das auf hohe Konservierung in DEV schließen lässt und das unabhängig vom Ursprung des Stammes oder seiner Virulenz ist. Des Weiteren wurden in 39 ORFs nicht-synonyme Nukleotid-Substitutionen entdeckt. Die verbleibenden 15 ORFs hatten Fragment-Insertionen bzw. –Deletionen, Verschiebungen des Leserahmens- oder nicht-synonyme Nukleotid-Substitutionen mit einem Effekt auf die Translations-Initiation oder Terminierung des jeweiligen Leserahmens. In 7 der 15 ORFs mit hoher, und 27 der 39 ORFs mit niedriger Variabilität wurden Polymorphismen ausschließlich in DEV 2085 gefunden, ein Befund, der wahrscheinlich im unterschiedlichen Ursprung dieses Stammes (Europa) gegenüber den drei ost-asiatischen Stämmen begründet ist. Fünf ORFs (UL2, UL12, US10, UL47 und UL41) mit Polymorphismen waren identisch zwischen dem virulenten DEV 2085 und CHv, unterschieden sich jedoch von den attenuierten Stämmen VAC oder Clone-03 und könnten potentielle DEV Virulenzfaktoren sein.

Die Ergebnisse dieser Arbeit zeigen, dass (1) das Fehlen von DEV gC zur Zunahme der Plaquegrößen *in* vitro führt, (2) gC eine Rolle in der Freisetzung des DEV spielt und (3) die Konstruktion eines infektiösen DEV Klons eine schnelle Generierung von Vektor-basierenden Impfstoffen zulässt. Dazu ergab (4) der Verglich des europäischen DEV-Stammes 2085 mit den ost-asiatischen DEV-Stämmen VAC, Clone-03 und CHv, dass die ORFs UL2, UL12, US10, UL47 sowie UL41 potentielle DEV-Virulenzfaktoren darstellen werden.

Summary

**Generation of an Infectious Clone of Duck Enteritis Virus (DEV) – A Basis for
Pathogenesis Studies and Vectored Vaccine Development**

This study focused on the construction of an infectious bacterial artificial chromosome (BAC) clone of duck enteritis virus (DEV) strain 2085 and efficient generation of a vectored DEV vaccine expressing hemagglutinin (H5) of high pathogenicity H5N1 avian influenza virus (AIV) based on this infectious clone. Subsequently, the whole genome sequences of DEV strain 2085 were determined and compared with the whole genome of DEV VAC strain and sequences of DEV Clone-03 and CHv.

For construction of the DEV infectious clone, the mini-F vector sequences were inserted into the genome of DEV strain 2085 by homologous recombination in lieu of the UL44 (gC) gene. DNA of the resulting in recombinant virus v2085-GFPΔgC was electroporated into *Escherichia coli* strain Megax and subsequently strain GS1783, and a full-length DEV BAC clone (p2085) was recovered. Transfection of p2085 into chicken embryo cells resulted in DEV-specific plaques exhibiting green autofluorescence under UV excitation, indicating the successful generation of an infectious clone of DEV strain 2085. A gC-negative mutant, v2085ΔgC, was generated by deleting mini-F vector sequences by using Cre-Lox recombination, and a revertant virus v2085ΔgC-R was generated by co-transfection of p2085 with UL44 sequences produced through PCR. Finally, AIV H5 sequences were inserted into p2085 to generate a vectored DEV recombinant virus for expressing of H5, and high-level H5 expression of the v2085_H5 virus was detected by indirect immunofluorescence and western blotting. Plaque size determination showed that the sizes of v2085ΔgC plaques were significantly bigger (12%) over those of parental 2085 virus or the v2085ΔgC-R revertant virus (ANOVA, $P<0.05$), while plaque areas of v2085_H5 or v2085-GFPΔgC were significantly decreased. No significant difference was observed between parental or revertant DEV and mutant or recombinant DEV with respect to virus titers determined after trypsinization titration of infected cells, while virus titers of infected-cell supernatants revealed significant reductions in case of the gC-negative viruses of more than 700-fold when compared to parental 2085 or v2085ΔgC-R. Cell-associated virus titers of gC-negative DEV also showed significant reduction of 50-500-fold than parental 2085 or revertant DEV (ANOVA, $P<0.05$).

The nucleotide sequence was derived from the 2085 genome cloned as an infectious bacterial artificial chromosome (BAC) clone. The DEV 2085 genome is 160,649 bp in length and encodes

78 predicted open reading frames (ORFs)(GenBank ID: JF999965), a number identical to that of the attenuated DEV VAC strain (GenBank ID: EU082088.2). Comparison of the genome sequences DEV 2085 and VAC with partial sequences of the virulent CHv strain and the attenuated strain Clone-03 was carried out to identify nucleotide or amino acid polymorphisms that potentially contribute to DEV virulence. No amino acid changes were identified in 24 of the 78 ORFs, a result indicating high conservation in DEV independently of strain origin or virulence. In addition, 39 ORFs showed only non-synonymous nucleotide substitutions. The remaining 15 ORFs had fragment insertion or deletions, frame-shift mutations or non-synonymous nucleotide substitutions with an effect on ORF initiation or termination. In 7 of the 15 ORFs with high and 27 of the 39 ORFs with low variability, polymorphisms were exclusively found in DEV 2085, a finding that likely is a result of a different origin of this strain (Europe) and the three other strains (Eastern Asia). Five ORFs (UL2, UL12, US10, UL47 and UL41) with polymorphisms were identical between the virulent DEV 2085 and CHv but different from VAC or Clone-03, and may, individually or in combination, represent DEV virulence factors.

We conclude that (1) absence of DEV gC results in increased plaque sizes in vitro, (2) gC plays a role in DEV egress, and (3) generation of an infectious DEV clone allows rapid generation of vectored vaccines, (4) DEV 2085 may represent an origin of Europe other than DEV VAC, Clone-03 and CHv of Eastern Asian, (5) UL2, UL12, US10, UL47 and UL41 may be related to virulence of DEV.

Chapter 1. Introduction

Duck viral enteritis (DVE) or duck plague (DP) is an acute, infectious and often lethal disease of many species of domestic and wild waterfowl such as ducks, geese and swans (Jansen and Wemmenhove, 1965; Kaleta et al., 2007; Keymer and Gough, 1986; Leibovitz, 1968; Montgomery et al., 1981). The causative agent of DVE is duck enteritis virus (DEV), which is informally classified in the *Alphaherpesvirinae* subfamily as Anatid Herpesvirus 1 denoted after the host family, *Anatidae*, by convention. After the first case of the disease was reported in Netherlands in 1923 (Baudet, 1923), it has been reported in many countries (Jansen and Kunst, 1964; Leibovitz and Hwang, 1968; Mukerji et al., 1965; Wobeser and Docherty, 1987).

1.1. Taxanomy and Morphology of DEV

DEV, being a member of the *Herpesviridae*, contains a double-stranded DNA. Mature DEV virions are spherical in shape and contain a nucleocapsid, tegument and envelope. Typical herpesvirus virions and nucleocapsids could be first detected in the spleen, thymus, and bursa of Fabricius (BF) 48 h after infection. The capsids were described to be approximately 93 nm in diameter with a core of appropriately 48 nm. Cytoplasmic virus particles at 48 h showed different morphology and sizes of 156-384 nm in diameter with a core of appropriately 75 nm (Breese and Dardiri, 1968; Tantaswasdi et al., 1988). When infected ducks die, many nucleocapsids, mature viruses, and viral inclusion bodies can be found in the nucleus and cytoplasm of infected liver, small intestine, spleen, thymus, and BF (Yuan et al., 2005).

Based on the morphologic criteria, DEV has for decades been informally classified in the *Herpesviridae* family, but not assigned to any genus according to the latest report of the International Committee on Taxonomy of Viruses (ICTV) released in 2009. The complete genome sequence of DEV VAC strains has been published in 2009 and composed of a unique long region, a unique short region, a unique short internal repeat and a unique short terminal repeat, indicating that the arrangement of the DEV genome is a class D. Locations and orientations of the seven conserved herpesvirus sequence blocks are the same as in other alphaherpesviruses (Pellett and Roizman, 2007). The length of the complete genome of DEV VAC is approximately 158-kbp, and the G+C content is 45%. Seventy-eight putative proteins are predicted to be encoded by DEV VAC (Li et al., 2009). Most of the open reading frames (ORFs) of DEV strains Clone-03, C-KCE and CHv were sequenced and published (Liu et al., 2009; Zhao et al., 2009). Davison and McGeoch submitted a proposal to ICTV on Feb. 3, 2010. They stated the description by Li et al. that the taxonomic position of DEV was osculant among the

Alphaherpesvirus genera was understandable. However, they argued that assignment of herpesvirus species to genera should be based on molecular phylogeny and not other genome characteristics. The authors also did protein sequence alignments for six well conserved genes (UL29, UL27, UL19, UL30, UL15 and UL28, respectively), resulting in a phylogenetic tree that showed that DEV fell into a clade represented by the *Mardivirus* genus. Finally the proposal recommended that DEV, taxonomically named Anatid herpesvirus 1, should be classified in that genus and added to the three existing members *Meleagrid herpesvirus 1*, *Gallid herpesvirus 2* and *Gallid herpesvirus 3*. This proposal is still up to the decision of ICTV.

1.2. Replication of DEV

Duck enteritis virus can replicate in duck embryo fibroblasts, duck embryo chorioallantoic membrane cells and duck embryo liver or kidney cells (Gough and Alexander, 1990; Jansen, 1961; Kocan, 1976). It is recommended to infect chorio-allantoic membranes of duck embryos for isolation of wild-type virus (Dardiri and Hess, 1968; Janson, 1968). DEV can be propagated in chicken embryos or chicken embryo cells (CECs) although it is usually not efficient for isolation of wild-type viruses. However, in some cases, DEV will develop cytopathic effect on CECs after direct inoculation with tissues samples (Kaleta et al., 2007). Viral intranuclear inclusion bodies can be found in DEV-infected cells (Hess and Dardiri, 1968). Cytopathogenic effect in the form of plaques can be formed on cells at 48-72 hours post infection (h p.i.) with DEV. According to electron microscopical observations, virions can be first observed in the nucleus and subsequently in both the nucleus and cytoplasm. Further observations show that enveloped virions in the cytoplasm are bigger than those in the nucleus, indicating the replication of virus DNA and formation of nucleocapsids take place in nucleus and that the envelope is obtained when nucleocapsids are translocated into the cytoplasm (Breese and Dardiri, 1968; Tantaswasdi et al., 1988). The peak of cell-associated and supernatant virus titers appear at 48 and 60 h p.i., respectively, when infectious doses of 1-2 tissue culture infectious doses 50 ($TCID_{50}$) per cell are used (Breese and Dardiri, 1968).

In vivo studies employing oral infection of DEV in domestic ducks showed that the virus can be detected primarily in the mucosa of the digestive tract, especially in the oesophagus as early as 24 h p.i., then spread to the BF, thymus, spleen kidneys, trigeminal ganglion, cloaca and liver. The principal site of virus replication are epithelial cells and macrophages of these organs, although virus antigen can be also found in lymphocytes (Islam and Khan, 1995; Shawky et al., 2000)

1.3. Pathology of Duck Plague

Disease symptoms may occur 3-7 days p.i. for domestic ducks and almost all of the ducks with apparent symptoms will die within 3-5 days. Adult blue-winged teal may die 63.5-68 hours after inoculation with virulent DEV, and contact animals within 161-162 hours after exposure. Canada goslings may die 119-133 hours after inoculation (Wobeser, 1987). The morbidity and mortality can vary from 5% to 100% and is dependent on host species and virus strain (Kaleta et al., 2007; Wobeser, 1987; Wobeser and Docherty, 1987). Disease can occur in ducks from 7 day-old ducklings to mature ducks and includes reduction in egg production in commercial or wild waterfowl (Walker et al., 1969; Wobeser and Docherty, 1987). Exposure of mallard and muscovy ducks to DEV may result in significantly reduced hatchability or even no hatching of eggs laid by carrier ducks (Burgess and Yuill, 1981). DEV can be detected in multiple systemic organs in the early phase of acute infection. The relatively high levels of DEV in small intestine and BF tissues of dead ducklings most likely reflect the abundance of target epithelial and lymphoid cells in these tissues (Qi et al., 2008).

The clinical course of disease normally is very rapid in many species. At the beginning of an outbreak in ducks or in some species like teal, clinical signs are very limited, often sudden death is the only sign. Along with the spreading of the disease in flocks, more clinical signs may be observed, such as elevation of the feathers on the dorsum of the head, neck and subsequently the whole body. Other clinical signs include lethargy, convulsions, ataxia, inability to stand, extreme thirst, loss of appetite, light-phobia, fluid exuding from bills, water-like or greenish diarrhea, and tremors of neck, head and the body (Wobeser, 1987).

The most observed gross lesions of duck plague are caseous plaques along the longitudinal folds of the esophagus, proventriculus and mucosal surface of the lower intestine, free blood or hemorrhage throughout the alimentary tract to the cloaca and epithelial necrosis with adherent membrane in cloaca. Limited lesions can be found in the rectum. Necrotic foci and/or hemorrhage and petechiae or ecchymoses in the liver can also be found in many cases of duck plague (Snyder et al., 1973). Hepatomegaly with petechial hemorrhages, petechial hemorrhages in the abdominal fat, petechial hemorrhages on the epicardial surface of the heart are gross lesions observed in ducks in some duck plague outbreaks (Davison et al., 1993). Almost all of the lymphoid tissues and organs are damaged to variable degrees. The spleen will become dark and change size in some cases. Yellow foci of necrosis or hemorrhages are visible on the surface or cut surface of thymus and clear yellow liquid around thymus can also be observed in some cases. Fibrinous core and mucosal hemorrhage are normally visible lesions of the BF, as

are mucosal hemorrhagic or necrotic bands over the intestinal lymphoid tissue circumscribing the intestine (Wobeser, 1987).

The most typical microscopic lesions include focal liver necrosis, necrosis of esophageal and intestinal mucosa, capillary hemorrhage, and intranuclear or intracytoplasmic inclusion bodies in tissues like the esophageal and cloacal epithelium (Barr et al., 1992; Davison et al., 1993; Montgomery et al., 1981; Wobeser, 1987). At the early stages of infection, microscopic lesions occur in the wall of vasculature, and obvious lesions can be observed in small blood vessels. Lymphoid tissues in the submucosa of the alimentary tract at the intestinal annular bands, esophageal-proventricular junction and in the cloaca, as well as that in the thymus, are undergoing cytolysis and necrosis. Necrosis of lymphocytes in the spleen can be found. Necrosis can be focal to massive with widespread hemorrhage and is usually found in the liver. Degeneration and/or necrosis of bile duct and pancreatic duct epithelium with intranuclear incusion bodies can be found. Follicles and lymphoid tissue in the BF are generally necrotic (Calnek and Barnes, 1997). The necrosis and also apoptotic degeneration of lymphocytes in thymus, BF, and spleen induced by DEV infection results may play an important role in the pathogenesis of DVE (Guiping et al., 2007).

Generally, variation in the presence or severity of clinical appearance and lesions of DVE can be observed in different epizootics, host ages, strains of the virus, sex of the birds, and different host species, especially between domestic waterfowl and migratory Anseriformes (Converse and Kidd, 2001; Kaleta et al., 2007; Leibovitz, 1968; Leibovitz and Hwang, 1968; Wobeser, 1987).

As a member of the herpesvirus family, DEV will establish latency. In white Pekin ducks sites for virus latency were determined as the trigeminal ganglia (TG), peripheral blood lymphocytes, spleen, thymus and bursa. Virus can be detected after reactivation of virus with dexamethasone treatment or a combination of dexamethasone and cyclophosphamide in latently infected ducks (Shawky and Schat, 2002). Mallard ducks could appear healthy, shed large amounts of virus, and did not seroconvert (Burgess and Yuill, 1983; Calnek and Barnes, 1997).

1.4. Epidemiology of Duck Plague

Duck plague was reported to occur in Europe as disease that is easy to distinguish from fowl plague by Jansen in 1968 (Janson, 1968). However, one case reported as fowl plague as early as 1923 in the Netherlands and another case diagnosed in Muscovy ducks with gross lesions similar to DP in the Republic of South Africa in 1948 both possibly were cases of duck plague. The first epizootic in the Americas was reported in 1967 in Pekin ducks in domestic duck farms

on Long Island (Leibovitz and Hwang, 1968), and serological evidence supported the exposure to DEV at three commercial farms on Long Island before 1967 (Newcomb, 1968). Even though strict control methods for DP including decontamination of the environment, quarantines, eradication of affected flocks and vaccination in flocks not known to have been infected were conducted to try to prevent this disease, this so called exotic disease was observed again in 1973 (Calnek and Barnes, 1997; Converse and Kidd, 2001). As early as 1957, the first case that was supposed to be duck plague in China was reported (Huang, 1959). Duck plague was also diagnosed in France, Belgium, India, Thailand, England, Canada, Denmark, Austria, Hungary, Germany and Vietnam (Calnek and Barnes, 1997).

A review collected 120 DEV epizootics reported from 1967 to 1995 in the United States. Duck plague epizootics occurred in 21 states and the greatest frequency of epizootics (86%) was found during March to June. At least 40 waterfowl species were affected, with the highest frequency in Muscovy ducks (Cairina moschata) (68%), mallard ducks (A. platyrhynchos) (18%) and black ducks (A. rubripes) (14%) (Converse and Kidd, 2001).

A DEV outbreak was reported during the movement of indoor farming of all captive birds from October 20 to December 15, 2005 to prevent avian influenza in Europe. A total of 17 out of 124 (14%) adult birds and 149 out of 184 1-year-old birds (81 %) died. It was discussed that disturbance of natural behavior, interruption of mating and breeding activities, and possibly additional stress following latent DEV infection in some birds are factors associated with the outbreak. In this case, 14 additional species of the order *Anseriformes* were found to be susceptible to DEV (Kaleta et al., 2007).

There are a variety of strains of DEV, some of which are more pathogenic than others, and susceptibility to the virus varies in different host species (Dardiri and Butterfield, 1969; Janson, 1968; Spieker, 1977). Rabbits, guinea pigs, mice, rats and adult pigeons are not susceptible to DEV under experimental conditions. Even though it has been proven that chickens up to two weeks of age are susceptible to DEV after serial passages in CEC, natural infection with DEV is limited to birds belonging to the *Anseriformes* (Janson, 1968). European teal (*Nettion crecca*) and the pintail (*Difila acuta*) are resistant to infection but can produce antibodies against DEV (Janson, 1968). To date, there are more than 48 species in the order *Anseriformes* reported to be natural hosts of DEV (Converse and Kidd, 2001; Davison et al., 1993; Janson, 1968). Species susceptible to DEV under natural or experimental infection include

Mandarin Duck (*Aix (Dendronessa) galericulata*);

Wood duck (*Aix sponsa*) ;

Egyptian goose (*Alopochen Egyptiacus*);

American Wigeon (*Anas American*);

Bahama Pintail (*Anas bahamensis*)

Green-winged Teal (*Anas crecca*);

Cinnamon Teal (*Anas cyanoptera*);

Blue-winged Teal (*Anas discors*);

Red-billed Pintail (*Anas erythrorhyncha*);

Mottled duck (*Anas fulvigula*);

Indian Runner Duck (*Anas platyrhynchos*);

Mallard ducks(*Anas platyrhynchos*);

Pekin duck (*Anas platyrhynchos domesticus*) ;

Black duck (*Anas rubripes*);

CommonShoveler (*Anas (spatula) clypeata*);

Chiloe Wigeon (*Anas (Spatula) sibilatrix*);

White-fronted goose (*Anser albifrons*);

Greylag goose (*Anser anser*);

Snow goose (*Anser(Chen) caerulescens*);

Bean goose (*Anser fabalis*);

Lesser scaup (*Aythya affinis*);

Redhead Duck (*Aythya Americanna*);

Tufted duck (*Aythya fuligula*);

Common Pochard (*Aythya ferina*);

Greater Scaup (*Aythya marila*);

New Zealand Scaup (*Aythya novaehollandae*);

Canvasback Duck (*Aythya valisineria*);

Canada goose (*Branta canadensis*) ;

Bamacle goose (*Branta leucopsis*);

Bufflehead duck (*Bucephala albeola*);

Goldeneye (*Bucephala sp.*);

Muscovy duck (*Cairina moschata*);

Ringed Teal (*Callonetta leucophrys*);

Gadwall (*Chaulelasmus strepera*);

Maned Duck (*Chenonetta jubata*);

Mute swan (*Cygnus olor*);

Black-bellied Whistling Duck (*Dendroxygna arborea*);

Wandering Whistling duck (*Dendrocygna arcuta*);

Red-billed Whilstling Duck (*Dendroxygna autumnalis*);

Plumed Whistling Duck (*Dendrocygna cytoni*);

Spotted Whistling Duck (*Dendrocygna guttata*);

White-faced Whistling Duck (*Dendrocygna viduata*);

Hooded Merganser (*Lophodytes cucullat*);

Hooded Merganser (*Lophodytes (Mergus) cucullatus*);

Widgeon (*Mareca penelope*);

European Wigeon (*Mareca (Anas) penelope*);

Commom Merganser (*Mergus merganser*);

Common pochard (*Nyroca ferina*);

Ruddy Duck (*Oxyura jamaicensis*);

Garganey teal (*Querquedula*);

Common eider (*Somateria mollisima*);

King Eider (*Somateria spectabilis*);

Shoveler (*Spatula clypiata*);

Commom shelduck (*Tadoma tadoma*);

Shell duck (*Tadorna tadorna*);

Ruddy Shelduck (*Tadoma (Casarca) ferruginea*)

1.5. Transmission of DEV

Susceptible birds can be infected by close contact to diseased birds under natural conditions. Indirect contact to contaminated environment such as ponds, moats and pools can also result in infection. It seems that infection of migratory waterfowl perhaps is a result of contact with infected non-migratory birds and vice versa. Sometimes, introduction of new birds may also cause an outbreak. During outbreaks, infected birds shed large amounts of virus, which become

new sources of contamination that will promote the epizootics to spread faster and wider. It was shown that reactivation of latent DEV perhaps is a crucial factor for triggering outbreaks of duck plague in some cases, for example under immunosuppression caused by disturbance of natural behavior and additional stress (Kaleta et al., 2007).

Following experimental infection, the disease can be provoked by means of intramuscular, oral, nasal, intravenous, intraperitoneal, cloacal or subepithelial administration or close contact with infected animals. DEV can also be transmitted to ducklings by vertical transmission that then also shed DEV (Burgess and Yuill, 1981 ; Montgomery et al., 1981)

1.6. Functions of DEV Proteins

There are 78 genes or ORFs predicted to be encoded by DEV. The gene products can generally be divided into proteins involved in DNA replication and nucleotide metabolism and virus structural proteins. Viral envelope (glyco)proteins are involved in interaction of DEV with host cells (Li et al., 2009). Up to date, only a limited set of gene products of DEV has been studied that include those encoded by UL55, UL53, UL51, UL45, UL38, UL31, UL24, UL15 and US7.

An analysis of the expression of UL55 of DEV through quantitative real-time PCR (qRT-PCR) was conducted. Briefly, the DEV UL55 gene sequences were amplified and cloned into plasmid pMD18-T vector, resulting in pMD18-T/UL55. Then, standard curves were established with DNA of this plasmid using pMD18-T/ß-actin DNA as a standard. Total RNA was isolated from mock-infected or DEV-infected cells at different time points from 0.5 to 60 h p.i. Subsequently the purified RNA was immediately inversed transcribed to cDNA, which was then used as a template to perform RT-PCR quantification with the standard curves established. The results suggested a low level expression of UL55 mRNA from 0 to 8 h post-infection (p.i.) and peak expression at 36 h p.i., indicating that this gene was produced most abundantly during the late phase of replication (Wu et al., 2011 -a; Wu et al., 2011 -b). The study on the expression of DEV UL53 (gK) gene and subcellular location of gK was done using qRT-PCR, nucleic acid inhibition test, western blotting and indirect immunofluorescence assays. The results showed gK was a late protein. The intracellular localization of DEV gK was shown to be in cytoplasm, which is coincident with properties of UL53 homologs of infectious laryngotracheitis virus (ILTV) and herpes simplex virus type 1 (HSV-1) (Zhang et al., 2010; Zhang et al., 2011). Research into the basic characteristics of pUL51 showed that palmitoylation of pUL51 at the N-terminal cysteine was required for its membrane and Golgi localization, and that pUL51 mainly was found at the juxtanuclear region of DEV-infected cells. The protein also seemed to be incorporated into mature virions as a component of the tegument (Shen et al., 2009).

The study of the UL45 gene showed that expression of UL45 mRNA was at a low level from 0 to 18 h p.i., then accumulated quickly at 24 h p.i., peaked at 42 h p.i., and can be detected until 72 h p.i. Besides, this study showed that the UL45 protein was located in purified virions (the viral envelope), indicating that the DEV UL45 gene is expressed with late kinetics (Shen et al., 2010a).

Western blotting and immunofluorescence results showed that pUL38 was expressed from 8 h post-infection with a diffuse distribution throughout the cytoplasm, and later in the nucleus. Furthermore, pUL38 was detected in purified virions as well (Xiang et al., 2010). qRT-PCR and Immunofluorescence analysis revealed that the DEV UL31 gene was transcribed most abundantly during the late phase of replication and the protein was widespread in speckled structures present in the nuclei of infected cells. Western blotting showed that pUL31 is a component of intracellular virions but was absent from mature extracellular virions. In vivo experiment showed that the pUL31 antigen was primarily located in the cells of digestive organs and immune organs such as the spleen, thymus and BF (Xie et al., 2009). Another experiment showed that pUL24 was predominantly nuclear membrane-associated, especially at later times post infection (Jia et al., 2009).

DEV UL15 is suspected to be a spliced gene that encodes two products encoded by 2.9 and 1.3 kb transcripts, respectively. The expression of DEV UL15 is found in the late phase of infection, and UL15 and/or UL15.5 accumulate (s) in the cytoplasm during earlier times p.i. and then are translocated to the nucleus at later time points (Zhu et al., 2011). A qRT-PCR showed that the DEV gI gene (orf 73) is transcribed most abundantly during the late phase of infection and that the protein was expressed and is located in the cytoplasm of the infected cells (Li et al., 2011).

Further studies are clearly needed for a complete understanding of DEV. To facilitate the research on molecular biological characteristics of the virus, the generation of a DEV BAC was successfully completed as outlined in chapter 2 of this thesis. The generation of a DEV gC deletion mutant was also described in this chapter, along with the properties of this mutant that can potentially serve as a modified live marker vaccine against duck plague.

1.7. Diagnosis of DEV

Infection with DEV can generally be diagnosed by the clinical signs, gross lesions and microscopic lesions, along with epidemiological observations. Nevertheless, a goose herpesvirus disease with similar clinical signs and gross lesions as those induced by DEV cannot easily be differentiated from duck plague in geese (Chen et al., 2008; Ketterer et al.,

1990) For detection of DEV viral DNA or protein as wells as antibodies against DEV, a number of different methods are available.

1.7.1. Serological assays

Plaque reduction and neutralization tests (NT) were developed as a standard diagnostic (serological) procedure for detection of DEV antibodies and have been in use for decades. Briefly, equal volumes of virus containing virus producing a sufficient number of plaques and complement-inactivated antiserum (with a final dilution of 1:10) are mixed and after co-incubation for 1 hour at 37 , the mixture is plated on fresh DECs or CECs to count the plaques. The extent of plaque reduction is equivalent to the neutralization effect of the serum tested. While it seems that there is a lack of positive correlation between virus neutralizing antibodies and the ability of vaccinated ducks to withstand challenge with virulent virus, this method can be used for detection of DEV as well (Butterfield and Dardiri, 1969; Dardiri and Hess, 1967; Dardiri and Hess, 1968; Shawky and Sandhu, 1997; Wolf et al., 1974).

Enzyme-linked immunosorbent assay (ELISA) is also a commonly used method for detection of antibodies against DEV. Briefly, DEV virus is purified and then used to coat ELISA plates overnight. Diluted serum samples are placed into DEV-coated wells for 45-60 min and finally detected using an enzymatic reaction. Absorbances are read and the results given relative to an established positive-negative cutoff value (Shawky and Sandhu, 1997)

An immunochromatographic strip (ICS) test was developed for detecting DEV serum antibodies. This test is based on membrane chromatography and purified recombinant UL51 protein. A test for specificity showed that only sera against DEV would yield strongly positive results. The sensitivity of the ICS test was almost consistent with that of an ELISA and much higher than NT. The assay is low cost, rapid (15 min) and easy to perform (Shen et al., 2010b).

1.7.2. Virus detection

The so-called reverse passive hemagglutination (RPHA) test was created to detect DEV in as early as 1984. This method uses sheep erythrocytes coated with immunoglobulin G containing anti-DEV antibody from antiserum produced in sheep. The specificity of the RPHA test is guaranteed by the fact that inhibition of hemagglutination only is observed if DEV antibodies are present. The RPHA test can get results only 3 h after incubation at 25 . RPHA can detect plaque-forming or non-plaque-forming strains of DEV. All the results showed that the RPHA test is a rapid, simple procedure with sufficiently sensitive for diagnostic detection of DEV in acute infections (Deng et al., 1984).

Originally, PCR assays were designed using primers located in the highly conserved domain of the UL6 gene and were used to successfully detect DEV DNA in diagnostic procedures. The PCR assay also amplified DEV DNA from samples of original outbreaks and/or after passage in Muscovy duck embryos (Plummer et al., 1998). Other PCR methods for the detection of DEV DNA were also developed (Pritchard et al., 1999 ; Zou et al., 2010). A qPCR assay was introduced for DEV DNA detection with the DNA polymerase gene as the target gene, which proved to be rapid, sensitive, and specific. This method can also be used for studies of pathogenesis of the virus (Yang et al., 2005). A loop-mediated isothermal amplification (LAMP) assay was developed and showed to be a simple, rapid, accurate, sensitive and specific method for detecting DEV that can be used if, for example, a PCR cycler is unavailable (Ji et al., 2009).

An antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) method was developed for the efficient detection of DEV pUL24 antigen. Tests showed that the AC-ELISA approach is rapid, sensitive, and reliable for the specific detection of DEV antigen (Jia et al., 2009). An indirect immunofluorescence (IIF) assay was established for the detection of the gE of DEV (Chang et al., 2011).

A common disadvantage of all diagnostic methods described above is that they cannot differentiate infection with DEV from vaccination. To determine the possible differences in open reading frames sequences or protein sequences between virulent strains and vaccines would provide choices to establish new diagnostic ways to overcome this difficulty. In chapter 3 of this thesis , the genome of the DEV 2085, a wild-type virulent strain of DEV, was determined and comparison with other virulent and vaccine strains was conducted. ORFs with sequences insertion/deletion, frame-shift were scrutinized to try to find factors related to virulence.

1.8. Vaccines against Duck Plague

Vaccination is the most important way for control of DEV. Both attenuated and inactivated vaccines with or without adjuvant can produce efficient protection, while vaccines inactivated with *beta*-propiolactone failed to induce strong immunity (Butterfield and Dardiri, 1969; Shawky and Sandhu, 1997).

After serial passage in chicken eggs or cells, the pathogenicity of DEV decreases rapidly and can resulting virus can still induce protective immunity in ducks against DEV. A DEV live vaccine in Holland attenuated through this method has been used for many years worldwide and has worked very well for control of the disease. An attenuated DEV vaccine (VAC strain) and a cloned strains (Clone-03 strain) have been used widely in duck flocks in China for decades (Li et

al., 2009; Liu et al., 2011). Inoculation with attenuated vaccine can produce protection as early as one day after application. Vaccination of exposed ducks and geese can result in immediate protection against disease development (Janson, 1968; Toth, 1970).

Attenuated vaccines seem to have high efficiency in almost all species of the *Anseriformes*. During the outbreak in 2005 in Germany, losses ceased immediately after the use of an attenuated DEV vaccine (Kaleta et al., 2007). However, two reports showed commercial DEV vaccine yielded poor immune response and only provided partial protection on challenge (Kulkarni et al., 1998; Mondal et al., 2010).

Research also showed that oral inoculation with attenuated DEV stimulated an IgA-dominant response in intestinal secretions. The virus-specific mucosal IgA had a peak at 15 days after inoculation, accompanied by the reduction of virus load in the intestine, suggesting that the mucosal IgA response has a role in controlling viral replication. But it is still not clear whether local immunity can also be induced by subcutaneous or intramuscular administration (Yang et al., 2010).

Significant numbers of virus genomes in the lymphoid and other parenchymatous organs were observed and rise to peak levels during 90 min to 1 day after vaccination, independently of the route of administration (subcutaneous, oral or nasal inoculation). However, the peak level of DEV in the individual parenchymatous organs of ducklings immunized subcutaneously was significantly higher than that of orally or nasally immunized animals. DEV spread to digestive tract and tracheal tissues earlier after oral and nasal inoculation than after subcutaneous administration. And after the rapid early increase of vaccine virus levels, a steady decline was observed from 90 min to 6 days post vaccination (Qi et al., 2009).

A study showed that duck embryos, even at 23-24 days of incubation, were capable of responding to infection of an apathogenic strain of DEV, which was injected into the allantoic cavity of duck embryos at 17 or 18 days of incubation, indicating the possibility of routine embryo vaccination for DEV (Lam, 2001). A nonpathogenic DEV strain (Sheridan-83) isolated from waterfowl was studied as a candidate for a modified live virus vaccine. Ducks inoculated with this virus developed resistance to challenge with the virulent strain LA (Lin et al., 1984). An inactivated vaccine of DEV was licensed in 2009 in China. The advantage of inactivated DEV vaccine is that it can circumvent the effect of maternal antibodies, which will neutralize live virus (Lin et al., 1984).

Conventional vaccines will induce immunity that cannot be differentiated conveniently from infections with wild-type virus. A gene-deleted marker vaccine would be a promising vaccine

candidate to make possible serological differentiation. In chapter 2 of the thesis, a gC deletion DEV mutant was constructed and its potential as a marker vaccine is discussed.

1.9. Ducks and their role in Avian Influenza Epidemiology

Currently, highly pathogenic avian influenza (HPAI) H5N1 viruses are still circulating in poultry in some countries and cause sporadic human infections that exhibit high lethality: Up to 30 April 2011, a mortality of 52 % of 552 confirmed human cases of infection with avian influenza H5N1 virus were reported. The threat of a future possible influenza pandemic has attracted much concern and promoted intensive research on this disease (Loeffelholz, 2010). The current WHO phase of pandemic alert for avian influenza H5N1 is 3 (http://www.who.int/en/).

Wild waterfowl are the natural reservoir of all known subtypes of influenza A viruses including HPAI H5N1 (Neumann et al., 2010). Wild ducks are the main reservoir of influenza A viruses. Several duck species are naturally resistant to highly pathogenic Asian H5N1 influenza with only few or no disease signs but shedding and spreading virus, indicating that ducks are the "Trojan horses" of H5N1 in their surreptitious spread of virus (Nazir et al., 2011; Kim et al., 2009). Recently, Some AIV H5N1 strains showed virulence in ducks and geese as well. Other findings suggested that ducks could generate H5N1 variants with novel amino acid substitutions leading to a potential change in the host range and allowed the virus to evolve into forms with increased pathogenicity (Cardona et al., 2009; Kim et al., 2011; Watanabe et al., 2011). One example is the H5N1 type A influenza virus classified as Qinghai-like virus (clade 2.2), a unique lineage of type A influenza virus with the capacity to produce significant disease and mortality in gallinaceous and anseriform birds, including domestic and wild ducks (Kwon and Swayne, 2010).

Because of the importance of ducks in the epidemiology of HPAIV H5N1, the potential of DEV to serve as a vector to express hemagglutinin of HPAIV H5N1 is worthy further research along with the capacity of herpesvirus as vector and the routine vaccination of DEV vaccines in duck flocks. In chapter 2 of this thesis, a DEV vector H5 recombinant was generated and evaluated in a proof-of-principle approach.

Generally, a concise list below can show the highlights of this thesis:

1. BAC construnction of DEV
2. Deletion of gC of DEV
3. Characterization of mutant virus
4. Vaccine production, including gC deleteion and H5 vectored vaccines

5. Sequencing of whole genome of DEV 2085

6. Comparison of genomes and gene sequences of DEV

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Chapter 2. Generation of an Infectious Clone of Duck Enteritis Virus and Generation of a Vectored DEV Expressing Hemagglutinin of H5N1 Avian Influenza Virus

2.1. Summary

We report on the generation of an infectious bacterial artificial chromosome (BAC) clone of duck enteritis virus (DEV) and a vectored DEV vaccine expressing hemagglutinin (HA) of high pathogenicity H5N1 avian influenza virus (AIV). For generation of the DEV BAC, we inserted mini-F vector sequences by homologous recombination in lieu of the UL44 (glycoprotein C, gC) gene of DEV isolate 2085. Deoxyribonucleic acid (DNA) of the resulting in recombinant virus v2085-GFPΔgC was electroporated into Escherichia coli and a full-length DEV BAC clone (p2085) was recovered. Transfection of p2085 into chicken embryo cells resulted in DEV-specific plaques exhibiting green autofluorescence. A gC-negative mutant, v2085ΔgC, was generated by deleting mini-F vector sequences by using Cre-Lox recombination, and a revertant virus v2085ΔgC-R was constructed by co-transfection of p2085 with UL44 sequences. Finally, AIV H5 was inserted into p2085, and high-level H5 expression of the v2085_H5 virus was detected by indirect immunofluorescence and western blotting. Plaque area measurements showed that v2085ΔgC plaques were significantly increased (12%) over those of parental 2085 virus or the v2085ΔgC-R revertant virus (ANOVA, $P<0.05$), while plaque areas of the H5- or GFP-expressing DEV mutants were significantly smaller. There was no significant difference between DEV with respect to virus titers determined after trypsinization titration of infected cells, while virus titers of infected-cell supernatants revealed significant reductions in case of the gC-negative viruses of more than 700-fold when compared to parental 2085 or v2085ΔgC-R. Cell-associated virus titers of gC-negative DEV also showed significant reduction of 50-500-fold (ANOVA, $P<0.05$). We conclude that (i) absence of DEV gC results in increased plaque sizes in vitro, (ii) gC plays a role in DEV egress, and (iii) generation of an infectious DEV clone allows rapid generation of vectored vaccines.

2.2. Introduction

Duck enteritis virus (DEV), taxonomically referred to as Anatid Herpesvirus 1 in the Alphaherpesvirinae subfamily of the family Herpesviridae in the order Herpesvirales, is the causative agent of duck plague, an acute and often fatal infectious disease of more than 48

species of waterfowl within the order Anseriformes, such as ducks, geese and swans. Morbidity and mortality vary but both can reach 100% in young and unprotected animals (Kaleta et al., 2007; Keymer and Gough, 1986; Leibovitz, 1968; Wobeser, 1987). Even though attenuated vaccines can provide protection against disease, infection is not prevented. Therefore, a better understanding of DEV pathobiology is certainly needed, as are novel vaccine candidates. Both, studying DEV biology and pathogenesis as well as vaccine development would benefit from the availability of an easy-to-manipulate genetic system, such as an infectious clone. After the mouse cytomegalovirus genome was first established as a 230 kilo base pair (-kbp) bacterial artificial chromosome (BAC) in E. coli (Messerle et al., 1997), the genomes of a number of herpesviruses have been cloned as BACs (Osterrieder et al., 2003; Rudolph et al., 2002; Smith and Enquist, 1999). BAC clones allow utilization of the large arsenal of bacterial mutagenesis protocols available for manipulation of the virus genome. Based on infectious herpesvirus clones, a large number of mutants have been generated to study viral pathogenesis and to develop (vectored) vaccines (Hocknell et al., 2002; Petherbridge et al., 2009; Rosas et al., 2007a; Rosas et al., 2007b; Rosas et al., 2008; Schumacher et al., 2000; Tsukamoto et al., 1999 ; Tsukamoto et al., 2002; Zhou et al., 2010).

Herpesviruses encode a number of glycoproteins that perform critical functions in the viruses' life cycles. In the case of the Alphaherpesvirinae, glycoprotein C (gC), albeit non-essential for propagation of all viruses studied so far, plays an important role in virus entry, egress, transmission and immune modulation . Studies on gC of Marek's disease virus (MDV), equine herpesvirus type 1 and 4 (EHV-1 and -4), pseudorabies virus (PRV), varicella-zoster virus (VZV), infectious laryngotracheitis virus (ILTV), feline herpesvirus type 1 (FHV-1) and herpes simplex virus type 1 (HSV-1) have shown that gC mediates virus attachment to target cells, is related to virulence and involved in virus release from infected cells (Adamiak et al., 2010; Azab et al., 2010; Herold et al., 1991; Jarosinski et al., 2007b; Jarosinski and Osterrieder, 2010; Johnson et al., 1986; Livingston et al., 2006; Mettenleiter et al., 1990 ; Moffat et al., 1998; Osterrieder, 1999; Pavlova et al., 2010; Rue and Ryan, 2008; Scheper et al., 2010; Storlie et al., 2008 ; Tischer et al., 2005; Trybala et al., 1993; Willemse et al., 1994). gC of EHV-1, HSV-1 or PRV also interact with the innate immune system by interfering with complement (Awasthi et al., 2009; Hook et al., 2008; Huemer et al., 1995; Huemer et al., 1993; Maeda et al., 2002; Stokes et al., 1991). So far, DEV has not been allocated to a genus within the Alphaherpesvirinae and identification of the functions of individual open reading frames may aid in such endeavors. In addition, generation of DEV gC deletion viruses may be exploited for the development of modified live (vectored) vaccines.

Epizootics and often deadly animal and human infections with avian influenza virus (AIV) H5N1 over the past decade have led to increased awareness and concern worldwide (Neumann et al., 2010; Peiris et al., 2007; Vijaykrishna et al., 2008). While wild aquatic birds including ducks are the main reservoir of influenza A viruses (Knipe and Howley, 2007) and many duck species will not succumb to the infection, H5N1 infections of some farmed duck species can have deadly outcomes. In addition, farmed ducks can serve as virus reservoirs and pose a constant threat to other poultry operations (Keawcharoen et al., 2008; Kim et al., 2009; Reperant et al., 2010; Sturm-Ramirez et al., 2005). Hence, control of AIV, currently primarily of H5N1, in ducks is crucial for disease control. DEV expressing hemagglutinin (HA) and/or neuraminidase (NA) of AIV may be promising candidates for parenteral or even oral vaccination of farmed or wild aquatic birds against AIV. Oral administration of DEV (vectored) vaccines is especially attractive, as it would make mass application feasible.

In this study we report on the generation of a system for DEV genome manipulation, which is based on an infectious BAC clone for the 2085 DEV strain that was recently isolated in Germany (Kaleta et al., 2007). Utilizing efficient mutagenesis protocols we were able to initially analyze the function of gC and could show it plays a major role in virus egress and seems to confer stability to the viruses during freeze-thawing. Finally, the potential of DEV to serve as vector system for expressing of H5 was evaluated and showed that high-level expression of foreign protein could be achieved.

2.3. Materials and Methods

2.3.1. Plasmids

Two pairs of primers, DEV-HOMO1-for/DEV-HOMO1-rev and DEV-HOMO2-for/DEV-HOMO2-rev (Table 2.1) , were designed to amplify the regions of the DEV genome flanking the UL44 gene encoding gC. Both fragments were approximately 1.0-kbp in size. The fragments were cloned into pUC19 (Invitrogen) using restriction enzyme sites present in the primers and the resulting recombinant plasmid was termed pUC19-I-II (Fig 2.1). A mini-F vector containing Eco-gpt and the GFP (green fluorescent protein) gene under the control of the immediate early promoter of human cytomegalovirus (P_{HCMV}) was released as a PacI fragment from plasmid pDS-pHA2 (Schumacher et al., 2000) and cloned into the PacI site present in pUC19-I-II to construct transfer vector plasmid pDEVgC-pHA2 (Fig 2.1). Plasmid pCAggs-NLS/cre expressing Cre recombinase was described earlier (Jarosinski et al., 2007b). Plasmid pEPgoinH5 was constructed as detailed earlier (Tischer et al., 2006) and contained P_{HCMV} sequences, the H5 gene and a kanamycin resistance gene in direct orientation and with appropriate flanking
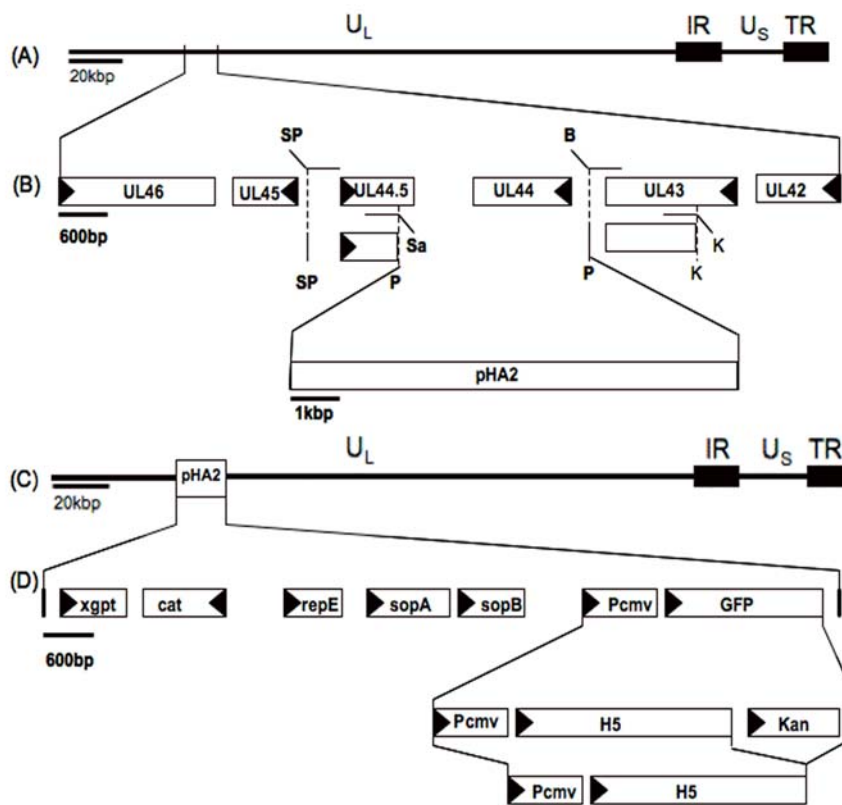
Figure 2.1. Construction of the DEV strain 2085 with mini-F sequences and the DEV-vectored H5 recombinant. (A) The organization of the ~160 kbp DEV VAC strain, which was used as a reference strain here, is shown. (B) Genomic organization from UL42 to UL46 of the unique long region of the DEV VAC strain was depicted. Two pairs of appropriate primers were employed to amplify two flanking fragments, then the mini-F-containing transfer vector was constructed by inserting pHA2 between the fragments in the correct direction. (C) After homologous recombination, pHA2 was inserted into the genome of DEV and the entire UL44 gene was deleted. (D) Schematic presentation of the DEV vectored H5 recombinant is shown. In a first step of Red recombination, the GFP cassette in pHA2 was replaced by H5 and Kan sequences, in the second step the kanamycin gene was deleted. Scales in base pair (bp) or kbp and restriction enzyme sites are provided. Sp, *Sph*I; B, *BamH*I; Sa, *Sal*I; P, *Pac*I; K, *Kpn*I.

sequences to allow replacement of the GFP gene present in pHA2 by homologous recombination. The H5 gene was synthesized as a codon-optimized version for eukaryotic expression (Geneart) and represented AIV A/Vietnam/1023/2004 (H5N1)(GenBank ID:AY 818135.1)(Fig 2.1).

2.3.2. Viruses, cells and transfection

DEV strain 2085 (kindly provided by Dr. Erhard Kaleta, Justus-Liebig-Universität Gießen), and mutants generated from this strain were prepared on primary or secondary chicken embryo cells (CEC), which were propagated in Earle's minimal essential medium (EMEM, Biochrom) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS) at 37°C under a 5% CO_2 atmosphere. Virus stocks were established from CEC cultures, which were infected with viruses at a multiplicity of infection (MOI). of 0.01. Infected cells were harvested by scraping and resuspension into 1 mL of EMEM-FBS. Viruses were released from cells by three freeze-thaw cycles (-70 °C and 37 °C) and were frozen in aliquots at -70 °C for

further use. Virus titers were determined by plaque assays on CEC. Briefly, 10-fold dilutions of virus were added to cells and allowed to attach and penetrate for 2 h at 37 $^{\circ}$C. The inoculum was then removed and replaced with fresh medium containing 0.5% (w/v) methylcellulose (Sigma-Aldrich). The number of plaques was counted under the microscope after 3 days of incubation. Viral Deoxyribonucleic acid (DNA) was purified from infected cells by sodium dodecyl sulfate (SDS)-proteinase K extraction as described earlier (Morgan et al., 1990). Transfection of plasmid, virus or BAC DNA was achieved using polyethyleneimine (Boussif et al., 1995) or calcium phosphate precipitation (Morgan et al., 1990).

2.3.3. Multi-step growth kinetics and plaque sizes determinations

Multi-step growth kinetics were conducted as described before (Adler et al., 2000; Rudolph et al., 2002) with some modification. Supernatant virus titers, cell-associated and virus titers of trypsinized cells were tested before infection and at 6h, 12h, 24h, 36h, 48h, 72h post-infection (p.i.) after infecting 1×10^6 CEC at an MOI of 0.01. For supernatant virus titers, 0.1 mL samples of supernatants of infected cells were collected at the indicated time points and centrifuged at 376XG for 5 min to remove cellular debris, then titrated on CEC. For cell-associated virus titers, infected cells were scraped from the plate and resuspended in 1mL EMEM-FBS after washing two times with phosphate-buffered saline (PBS). Viruses were released from cells by three freeze-thaw cycles and titrated on fresh CEC. For titration of cell-associated virus titers, infected cells were removed from plates using 0.05% trypsin which can separate CEC into individual cell without lethal damage and were titrated on fresh CEC. Three independent experiments were conducted for determining growth kinetics. One-way ANOVA (SPSS software package) was employed to determine significant differences (Saksena et al., 2006; Suchodolski et al., 2009).

Plaque sizes were measured at 48 h after plating 50 plaque forming units (PFU) of the respective viruses onto 1×10^6 CEC seeded in a well of a 6-well plate and after adding medium containing 0.5.% methylcellulose 2 h after plating. Indirect immunofluorescence (IIF, see below) was performed to visualize the plaques. For every virus, 150 plaque images were taken using a digital camera (Axiocam, Zeiss) mounted on an inverse fluorescence microscope (Axiovert S100, Zeiss). ImageJ software (NIH)(http://rsbweb.nih.gov/) was employed for the measurement of the plaque areas. One-way ANOVA was again employed to analyze the differences of plaque sizes between different strains.

2.3.4. Bacterial manipulations

Chemically or electrocompetent *E. coli* cells Top10 (Invitrogen), MegaX (Invitrogen) or GS1783 (Jarosinski et al., 2007a; Lee et al., 2001; Tischer et al., 2010) were used. The cells were

provided by a commercial supplier (Top10, MegaX) or prepared exactly following previously described protocols (Muyrers et al., 1999; Narayanan et al., 1999). Preparation of DNA of plasmid or BACs was achieved with commercial kits (Qiagen). Electroporation was used to introduce viral or BAC DNA and methods as described earlier were followed (Schumacher et al., 2000; Shizuya et al., 1992; Tischer et al., 2006) with slight modifications. Briefly, electroporation was performed in 1-mm cuvettes at 1,500 V/cm, a resistance of 200, and a capacitance of 25 F (Genepulser Xcell, Biorad). Transformed bacteria were incubated in 1 mL of SOC medium or Luria-Bertani (LB) medium for 1 h at the proper temperature and then plated on LB agar containing proper antibiotics and incubated at the required temperature.

2.3.5. Generation of a DEV BAC clone

For generation of the DEV BAC clone, a mini-F containing DEV mutant was constructed following the general technique of BAC construction described before(Messerle et al., 1997; Schumacher et al., 2000) with some modifications. Briefly, approximately $1X10^6$ cells CEC were transfected with approximately 1 µg 2085 infected-cell DNA and 1 to 10 µg pDEVgC-pHA2 DNA (Fig 2.1). When cytopathic effect (CPE) was observed, the transfection culture was harvested by trypsinization, infected cells diluted, plated on fresh CEC, and overlaid with EMEM-FBS containing 0.5% methylcellulose. When green fluorescent plaques were identified, plaque-purification was carried out as described earlier (Rudolph et al., 2002) to obtain a homogeneous population. Mini-F containing DEV mutant viruses were propagated on fresh CEC. At 3 days p.i., viral DNA was isolated and then transferred into *E.coli* MegaX cells by electroporation. BAC DNA was isolated and restriction fragment length polymorphisms (RFLP) were determined as described before (Schumacher et al., 2000) using *EcoR*I and *Bgl*I. The whole genome sequence of DEV (VAC) was used as a reference (GenBank ID: EU082088.2) for *in silico* predictions using VectorNTI (Invitrogen). Finally, DEV BAC DNA was electroporated into *E.coli* strain GS1783 cells (Tischer et al., 2010) for mutagenesis.

To repair the gC gene in the gC-negative virus, homologous recombination was used to replace the mini-F vector in the DEV genome with the deleted sequences which were amplified by polymerase chain reaction (PCR) using 2085 DNA as a template and primer pairs of DEV-HOMO1-for and DEV-HOMO2-rev (Table 2.1). Non-fluorescent plaques were purified to obtain a homogeneous virus population.

To generate gC deletion mutants of DEV without vector sequences, Cre/Lox recombination was performed as described before (Chang and Barry, 2003; Jarosinski et al., 2007a; Smith and Enquist, 2000; Yu et al., 2002). Briefly, CEC were transfected with 1 to 2 µg DNA of pCAggs-

NLS/cre using calcium phosphate precipitation and cells were infected 12 h later with 50 to 100 PFU of DEV mutant viruses obtained after transfection of p2085. Non-fluorescent virus plaques were isolated and purified to homogeneity.

For manipulation of the DEV BAC, two-step Red (*en passant*) recombination was performed to replace GFP gene of the mini-F sequences with a HA gene (Fig 2.1) exactly as described before (Tischer et al., 2010; Tischer et al., 2006). Briefly, PCR was done with primer pairs HA5-for and HA5-rev (Table 2.1) using pEPgoinH5 as a template and LongAmp *Taq* polymerase (NEB). After digestion of the PCR product with *Dpn*I, 0.1 µg of gel purified PCR product was electroporated into electrocompetent GS1783 containing p2085. Electroporated cells were plated on LB plates containing chloramphenicol (34µg/mL) and kanamycin (50µg/mL) and incubated at 32 °C for 2 days. Double-resistant colonies were selected for the 2nd Red recombination to remove the kanamycin resistance gene. Colonies resistant to chloramphenicol but sensitive to kanamycin were selected. Subsequently, RFLP were determined after digestion with endonucleases *Bam*HI or *Sac*I.

2.3.6. Diagnostic PCR and sequencing

To prove presence or absence of gC in the obtained mutants, diagnostic PCR and fragments nucleotide sequencing were performed with the original wild type virus as control. A pair of primers specific for gC of DEV (gC_SEQ-for2 and gC-SEQ-rev2) and another pair of primers specific for homologous sequences upstream and downstream of DEV UL44 (DEV-HOMO1-for and DEV-HOMO2-rev) were used to perform the PCR (Table 2.1). Nucleotide sequencing of the gC gene was done using a set of 6 primers (Table 2.1) following the protocol of the service supplier (StarSEQ). To assess correct insertion of H5 into the DEV BAC, PCR was performed with primers of H5-SEQ-for1 and H5-SEQ-rev1 (Table 2.1). Nucleotide sequencing of the obtained recombinants using 8 specific primers (Table 2.1) was performed to check the correct insertion of H5.

2.3.7. Indirect immunofluorescence (IIF) and western blotting

For identification of DEV plaques, a method described earlier was used with some modifications (Seyboldt et al., 2000). Antibodies against DEV obtained from ducks (kindly provided by Dr. Erhard Kaleta, Justus-Liebig- Universität Gießen and Dr. Kathrin Bergmann, Lohmann Animal Health) were employed as primary antibodies and mouse-anti-duck IgG monoclonal antibodies (kindly provided by Dr. Bernd Kaspers, LMU München, Germany) were used as secondary

antibodies (Kothlow et al., 2005). The tertiary antibody used was Alexa Fluor® 568 Goat-anti-mouse IgG (H+L) (Invitrogen).

IIF of H5 was conducted with AIV H5-specific monoclonal antibodies (Rosas et al., 2006; Rosas et al., 2008) as described earlier. Briefly, CEC were grown on 6-well plates and subsequently infected with v2085_H5 (50 to 100 PFU per well). At 48 h after infection, cells were fixed and IIF was performed as described before (Rudolph et al., 2002; Seyboldt et al., 2000) with a mixture of monoclonal antibodies against H5 as the primary antibodies and goat-anti-mouse IgG antibodies conjugated with Alexa488 (Invitrogen) as the secondary antibodies.

To check HA expression of v2085_H5, western blot analysis was carried out as described before (Oh et al., 2010) with modifications. Briefly, CEC were infected with the v2085_H5 virus at an MOI of 0.01. At 48h p.i, cell lysates were prepared (Seyboldt et al., 2000) Lysates of cells infected with v2085-GFP∆gC were used as controls. Denaturation was carried out by heating at 95^oC for 10 min. Where indicated, removal of N-linked glycans was achieved by digestion with PNGaseF (NEB) at 37^oC for 10 min according to the supplier's instructions before loading the samples. Proteins were separated by SDS-10% polycarylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Macherey&Nagel) by the semi-dry method (Seyboldt et al., 2000). The same mixture of H5 antibodies used for IIF were also used for western blotting. We used a 1:2,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) as the secondary antibody and detection was done using enhanced chemoluminescence (Sigma-Aldrich).

2.4. Results and Discussion

2.4.1. Generation of mutant DEV strain 2085 harboring mini-F plasmid sequences

Initially, 1-kbp fragments flanking the gC gene (UL44) were amplified by PCR. The sequences upstream and downstream of the gC gene were cloned into a standard cloning vector before mini-F vector sequences were inserted between the flanks (Fig 2.1). Upon successful cloning, the transfer vector pDEVgC-pHA2 (~4.0 µg) and viral 2085 DNA (~1.5 µg) were co-transfected into CEC. Four days after transfection, CPE was observed. The transfected cells were harvested by trypsinization and serial dilutions were plated on fresh cells with the goal to obtain a homogeneous population of green plaques (Fig 2.2). After three rounds of plaque purification, a homogeneous population of fluorescing 2085 gC deletion viruses were obtained and termed v2085-GFP∆gC.

Table 2.1. Primers for PCR or nucleotide sequencing

Primer	Sequence
DEV-HOMO1-for	5'-*GCA*ggtacc*CAAGAACGGAGGTCAAGACAGA-3'[a]
DEV-HOMO1-rev	5'-*GAT*aagtcc*CT*ttaattaaCGTACTGTTGCAGTCAGTGTT-3'[a]
DEV-HOMO2-for	5'-*GGCG*gtcgac*AG*ttaattaaGCAAATACGCCAAGTCGCTTTG-3'[a]
DEV-HOMO2-rev	5'-*GAA*gcatgcGTTCGGAGACTGAAAACTCGTC-3'[a]
gC-SEQ-for1	5'-GCGTCCCGAATATAATGAATGCTAT-3'
gC-SEQ-for2	5'-GCATGGCCCGATTCGAATGTTAAG-3'
gC-SEQ-for3	5'-CGGTCGGTTCAAACCCATAATG-3'
gC-SEQ-rev1	5'-GCACATAGTCTTCGGCCGGT-3'
gC-SEQ-rev2	5'-CGACTGCTCTAGACGAGGGATTGTA-3'
gC-SEQ-rev3	5'-GACACAGCTGCGGTTGGAATT-3'
HA5-for	5'-AACCGGGCTGCATCCGATGCAAGTG-3'
HA5-rev	5'-GATCTGCTAGATAACTTCGTATAATGTATGCTATAC-3'
HA5-SEQ-for1	5'-CTGTTCGCCATCGTGAGCCT-3'
HA5-SEQ-for2	5'-CCCACCATCAAGCGCAGCTA-3'
HA5-SEQ-for3	5'-CGAGAGGCGCAGGAAGAAGC-3'
HA5-SEQ-for4	5'-TACAGCGAGGAAGCCAGGCT-3'
HA5-SEQ-rev1	5'-TGCACATCCACAGGGACAGG-3'
HA5-SEQ-rev2	5'-TGGGTGCTCTCTTTGTCGGC-3'
HA5-SEQ-rev3	5'-TCTGGTTCAGGGTGCTGGTG-3'
HA5-SEQ-rev4	5'-TGGGTCTTTTCCAGGATGTCC-3'

[a]:sequences in italics indicate additional bases which are not present in the DEV sequence and restriction enzyme sites are in bold lower case letters.

2.4.2. Generation of infectious DEV BAC clone p2085

For generation of a full-length DEV BAC clone, DNA of CEC infected with v2085-GFPΔgC was isolated after complete CPE had developed. Approximately 5 μg of infected-cell DNA was electroporated into *E. coli* MegaX cells (Invitrogen). Several chloramphenicol-resistant colonies were obtained 2 days after plating of electroporated cells and RFLP were determined to confirm that a full-length 2085 BAC clone was indeed generated. DNA of two selected colonies (S1 and S2) was isolated and digested with restriction endonucleases *EcoR*I or *Bgl*I. When RFLP pattern of S1 and S2 were compared to *in silico* predictions, which were based on the wholegenome sequence of a vaccine DEV strain (Li et al., 2009), the obtained *EcoR*I patterns of S1 and S2 matched those of the predictions well. In contrast, the results of the *Bgl*I digest did not entirely correspond to the predicted pattern (Fig 2.2). The results indicated that there were some differences between the sequenced DEV vaccine strain originating in China and the European wild-type strain 2085 used here. An alternative explanation, albeit unlikely, was that two apparently identical BAC clones that only contained parts of the 2085-GFPΔgC genome were obtained. Ultimately, S1 DNA was electroporated into *E. coli* strain GS1783 and the resulting
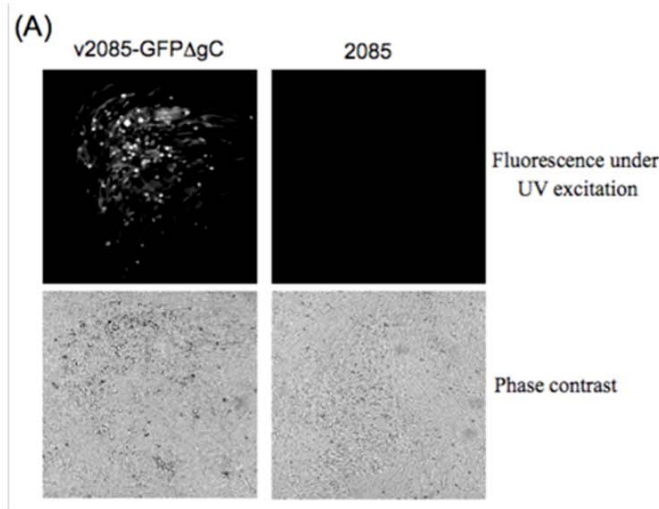
Figure 2.2. Plaque formation by v2085-GFPΔgC, RFLP of constructed DEV BAC clones S1 and S2, and PCR analysis of gC-deleted DEV and revertant virus.

(A) Plaques of recombinant 2085-GFPΔgC and parental 2085 virus. Plaques are shown under UV excitation (upper panels) or phase contrast (lower panels). 2085-GFPΔgC plaques were autofluorescent, while those of 2085 were not. Individual panels represent views of 600×600 μm. (B) DNA of DEV BAC clones S1 and S2 was isolated and digested with the indicated restriction enzymes. Digests were separated by 0.8% agarose gel electrophoresis at 40V for 17h. (C) Predicted RFLP patterns are shown with size markers from 2 to 20kb. Lanes1,3: digestion with *Bgl*I of S1 and S2, respectively, Lanes 2,4: digestion with *EcoR*I of S1 and S2, respectively. Predicted patterns of digestion with *Bgl* I and *EcoR*I were done using DEV(VAC) as reference. Lane M: Qick-Load® 1kb DNA Ladder(NEB). (D) PCR analysis of 2085, v2085ΔgC and v2085ΔgC-R are shown. Lanes 1-3 are PCR amplifications products of 2085, v2085ΔgC and v2085ΔgC-R respectively with primer pairs gC-SEQ-for2 and gC-SEQ-rev2. Lanes 4-6 contain PCR products of 2085, v2085ΔgC and v2085ΔgC-R, respectively, with primer pairs of DEV-HOMO1-for and DEV-HOMO2-rev. Lane M: GeneRuler[TM] 1kb Plus DNA Ladder (Fermentas).
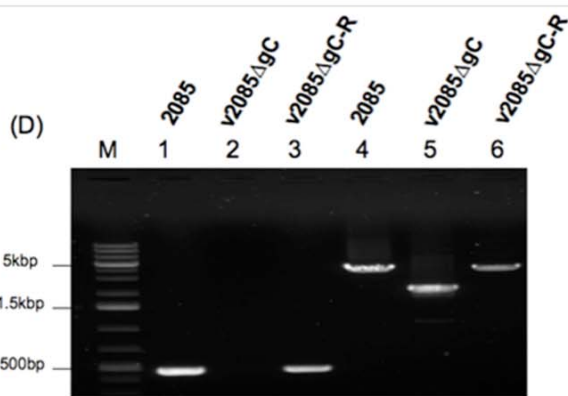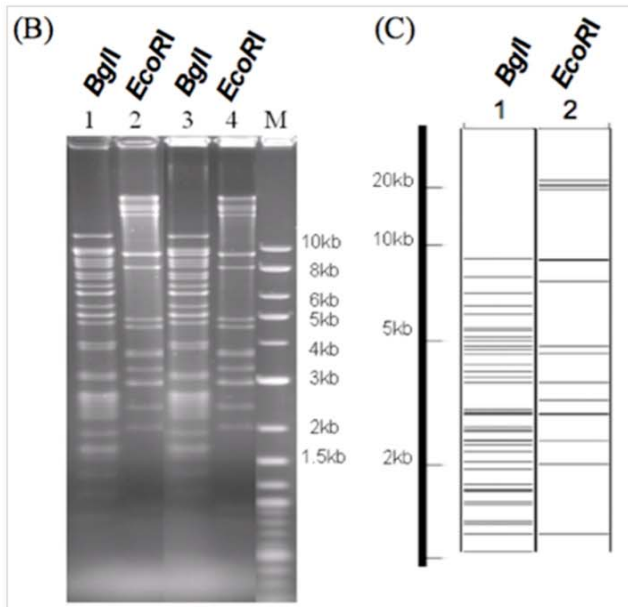
clone was termed p2085. All further experiments were conducted with p2085 present in GS1783 cells, which contain the necessary recombination systems needed for two-step Red mutagenesis (Tischer et al., 2010; Tischer et al., 2006).

2.4.3. Generation of p2085_H5

Based on p2085 and as a proof-of-principle approach, we sought to insert H5 of high-pathogenicity AIV A/Vietnam/1023/2004 into mini-F vector sequences. We targeted the GFP sequences present in the vector (Tischer et al., 2010; Tischer et al., 2006). With a first Red recombination, 100 ng of gel-purified PCR product was electroporated into GS1783 cells harboring p2085. The PCR product was obtained from plasmid pEPgoinH5 that contains a codon-optimized version of H5 under the transcriptional control of the P_{HCMV} using primers H5-for and H5-rev (Table 2.1) that specified the appropriate flanking sequences to allow recombination to replace the GFP gene with the H5 expression cassette. Numerous colonies with resistance to chloramphenicol and kanamycin introduced with the transfer cassette (Fig 2.1) were obtained 2 days after plating. One clone was selected to perform the 2nd Red recombination, which resulted in removal of the kanamycin resistance gene and the final H5-containing construct, termed p2085_H5 (Fig 2.1). PCR analysis showed bands of the expected size in p2085_H5, and RFLP patterns after digestion with *Bam*HI and *Sac*I corresponded well with the predicted pattern after insertion of the approximately 1.7-kbp H5 sequence. After digestion with *Bam*HI, p2085_H5 missed a band of approximately 12.2-kbp observed in p2085 and, instead, two bands of 7.5-kbp and 5.7-kbp were present. After digestion with *Sac*I, p2085_H5 missed a band of 20.5-kbp relative to p2085 and instead specified two bands of 13.8-kbp and 7.7-kbp, respectively (Fig 2.3). The results of the RFLPs were confirmed by PCR amplification of the insert and nucleotide sequencing, which demonstrated correct insertion of H5 sequences that did not show any nucleotide exchanges after quite comprehensive molecular manipulation. We therefore concluded that we had successfully cloned a H5 recombinant clone based on the DEV BAC that was used for further analysis.

2.4.4. Reconstitution of recombinant DEV from cloned DNA and generation of mutant viruses

One day after transfection of p2085 DNA into CEC, green fluorescent plaques were beginning to appear. The plaques became enlarged and more readily detectable after 2 days, ultimately demonstrating that replicating DEV could be reconstituted from the p2085 BAC clone. The reconstituted virus still expressed GFP and was devoid of gC. In order to generate a gC deletion mutant without a reporter gene, Cre/Lox recombination was used to remove mini-F vector sequences from the recombinant virus. To achieve removal of the mini-F sequences, v2085-GFPΔgC was used to infect CEC that had been transfected with pCAggs-NLS/cre DNA 12 h before. Following Cre-mediated removal of mini-F vector sequences, non-fluorescing plaques
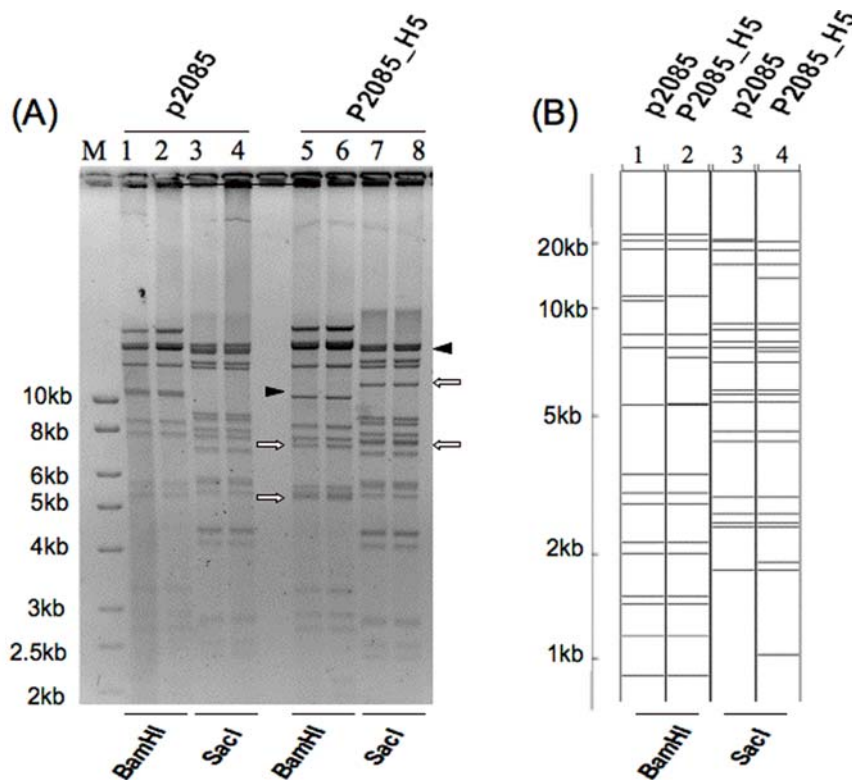
Figure 2.3. Restriction fragment length polymorphism (RFLP) analysis of p2085_H5).

(A) RFLP pattern of p2085_H5 and p2085. M is 1kb DNA marker (AllStar Scientific), lanes 1 and 2 are two replicas of p2085 digested with *Bam*HI; lanes 3 and 4 are two replicas of p2085 digested with *Sac*I; lanes 5 and 6 are two replicas of p2085_H5 digested with *Bam*HI, lanes 7 and 8 are two replicas of p2085_H5 digested with *Sac*I. The arrowhead (lane 5) shows a band of 12.2 kb missing, while the two arrows below show two new bands of about 7.5kb and 5.7kb. The arrowhead (lane 8) shows a band of 20.5 kb missing and the two arrows below show two newly arising bands of about 13.8kb and 7.7kb. (B): Predicted RFLP pattern of p2085_H5 compared to p2085. Lane 1, Prediction of p2085 digested with *Bam*HI; Lane 2, prediction of p2085_H5 digested with *Bam*HI; lane 3, prediction of p2085 digested with *Sac*I; lane 4: prediction of p2085_H5 digested with *Sac*I. The sequence of reference strain DEV (VAC) was used for the predictions.

appeared, which were picked and purified to obtain a homogeneous, non-fluorescing population. The resulting virus was termed v2085ΔgC and lacked gC and mini-F sequences as shown by PCR analysis (Fig 2.2) and nucleotide sequencing of the modified genetic region.

DNA of p2085_H5 was transfected into CECs to reconstitute an infectious virus, which was termed v2085_H5. The recombinant virus v2085_H5 specified non-fluorescing plaques as expected and was used for further characterization.

The gC revertant virus was obtained by co-transfection of p2085 DNA with DEV UL44 and flanking sequences containing a fragment that was amplified by PCR with primers DEV-HOMO1-for and DEV-HOMO2-rev (Table 2.1). The fragment allowed homologous recombination for re-insertion of gC coding sequences. Non-fluorescing plaques were picked and purified to obtain a homogeneous population. PCR amplification and nucleotide sequencing revealed presence of UL44 sequences in the engineered revertant virus (Fig 2.2) , which was termed v2085ΔgC-R. From our results we concluded that the p2085 BAC clone was infectious and that removal of mini-F sequences by Cre/Lox recombination yielded a virus that had a deletion in the

gC gene. Also, a gC revertant virus and a H5-vectored recombinant of 2085 were constructed successfully.

2.4.5. Robust expression of AIV HA by recombinant DEV v2085_H5

Indirect immunofluorescence and western blot analysis was performed with a mixture of monoclonal antibodies raised against AIV H5 (Rosas et al., 2008). Plaques of v2085_H5 reacted well and strong signals could be visualized using Alexa Fluor® 568-conjugated goat-anti-mouse IgG antibodies (Invitrogen). In contrast, plaques of v2085-GFPΔgC that was used as a control did not react with the antibodies. In western blots, proteins with apparent molecular masses of 80 kilo Dalton (-kDa) and 55-kDa were detected in lysates of cells infected with v2085_H5, whereas no reactive protein band was detected in CEC infected with v2085-GFPΔgC (Fig 2.4). We concluded that the 80-kDa moiety reactive with the specific antibodies represented the uncleaved HA precursor (HA0) as described before(Mayrhofer et al., 2009), whereas the faster migrating band represents the HA1 subunit that is produced after cleavage of HA0. After enzymatic deglycosylation using PNGaseF, an enzyme removing all N-linked carbohydrates from polypeptides, a weak band with an apparent molecular mass of approximately 68-kDa was observed. We concluded that this band represented the uncleaved HA0 precursor after removal of N-linked glycans, because it exhibited a size that corresponded well to that predicted from the primary amino acid sequence. Additionally, a band with a molecular mass of around 40-kDa, likely representing non-glycosylated HA1, in v2085-H5 lysates was detected after PNGaseF digestion. From our results we concluded that the recombinant v2085_H5 DEV can efficiently express H5 from a highly pathogenic AIV and that the presence of the polybasic cleavage site is utilized after expression in CEC. We also surmise that the reduced reactivity of the detected bands after enzymatic deglycosylation is caused by reduced affinity of the used monoclonal antibodies with the non-glycosylated form of the protein. In addition, none of the antibodies was apparently able to recognize HA2 subunit. From what we observed we can deduce that DEV has the potential to serve as a vector vaccine against AIV as high-level and stable expression of H5 was demonstrated. Immune effect in ducks will be tested in the future with the insertion of the H5 expression cassette in a way without the mini-F vector, along with the gC-deleted DEV replication properties in ducks.

2.4.6. Growth properties of recombinant DEV

Growth properties of the engineered mutant and revertant viruses were compared to those of the parental DEV strain 2085. Plaque areas were determined by IIF using anti-DEV polyclonal
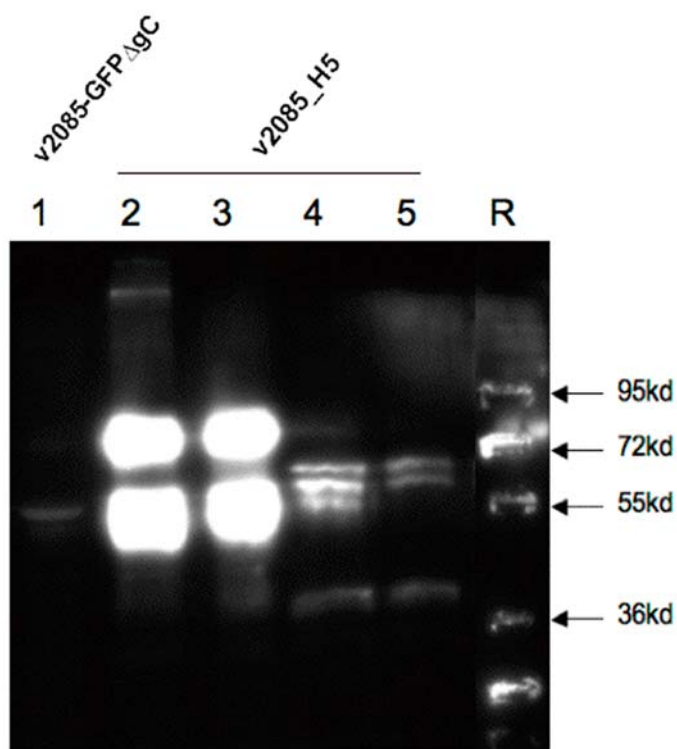
Figure 2.4. Western blot analysis for HA expression of v2085_H5 in CEC. Lane 1 contains lysates of CEC infected with v2085-GFPΔgC as a negative control, lane 2 contains lysates of CEC infected with v2085_H5 without denaturation (2-mercaptothanol) and deglycosylation (PNGaseF). Lane 3 was lysates of CEC infected with v2085_H5 with denaturation but without deglycosylation. Lane 4 contains deglycosylated lysates of CECs infected with v2085_H5 without denaturation, while in lane 5 deglycosylated lysates of CEC infected with v2085_H5 with denaturation were separated. In lane R, the size marker was run (PageRuler™ Plus, Fermentas).

antibodies obtained from ducks experimentally infected with 2085 (Kaleta et al., 2007). Mouse anti-duck IgG monoclonal antibodies were used as secondary antibodies and, finally, goat anti-mouse IgG (H+L) antibodies conjugated with Alexa488 (Invitrogen) were used as the tertiary antibody. When plaque areas of mutant viruses were compared to those of parental 2085 virus, it was discovered that plaque sizes of v2085ΔgC were significantly increased relative to the wild-type virus at 48 h p.i., when a 12% increase was noted (P=0.003). In contrast, v2085-GFPΔgC and v2085-H5 viruses exhibited significantly reduced plaque areas when compared to parental 2085 virus (P=0.0001 and p=0.029, compared to 2085, respectively). This included the 2085-GFPΔgC virus that also does not harbor UL44 sequences but expresses GFP instead. As expected, however, there was no significant difference between 2085 and v2085ΔgC-R plaque areas (P=0.424)(Fig 2.5). We concluded from the plaque area determinations that the absence of gC increases the efficiency of cell-to-cell spread of DEV, at least before 48 h p.i. The enhanced cell-to-cell spread, however, is offset if DEV expresses GFP or H5 (Fig 2.6). The result of increased plaque sizes after deletion of UL44 was intriguing inasmuch as similar observations were made with another avian alphaherpesvirus, MDV, in which gC was shown to be detrimental for virus growth. In the case of MDV the effect is attributed to secretion of gC and the binding of soluble gC to cognate receptors (Tischer et al., 2005). It is tempting to speculate that a portion of DEV gC may also secreted; however, a DEV gC- specific antibody is not
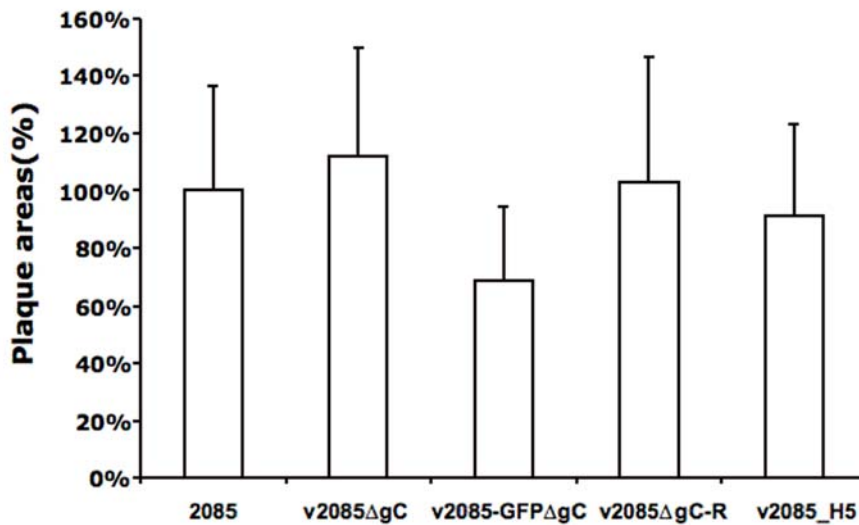
Figure 2.5. Plaque area measurement of 2085, v2085ΔgC, v2085-GFPΔgC, v2085ΔgC-R and v2085_H5 on CEC. Means and standard deviations of sizes of 150 plaques of each strain were measured with ImageJ software. The Mean of plaque areas of 2085 was set at 100%. Standard deviations are shown with the error bar.

available to us and investigations on the nature and potential secretion of DEV gC will be conducted in the future.

In the next series of experiments, we determined multi-step growth properties of the various mutant viruses and the differences of titers at 48h and 72h p.i. were statistically analyzed. v2085ΔgC, v2085-GFPΔgC and v2085-H5 titers in supernatants of infected CEC showed a marked reduction of more than 700-fold when compared to those observed after infection with parental 2085 virus or the v2085ΔgC-R revertant virus (p=0.001 for all, compared to 2085 at 48h and 72h) . No significant differences were observed between the latter two viruses at 48h (p=0.105), but it showed a significant difference at 72h (p=0.018), while there were also significant differences at 72h between v2085ΔgC, v2085-GFPΔgC, v2085-H5 and v2085ΔgC-R (p=0.005 for all)(Fig 2.6A).

The cell-associated virus titers at 48h p.i. determined after three freeze-thaw cycles largely paralleled those observed in the supernatants, although the reduction in virus titers after deletion of gC was not quite as striking. A significant 50- to 500-fold reduction of virus titers was observed for v2085ΔgC, v2085-GFPΔgC and v2085-H5 when compared to parental 2085 or the gC revertant virus v2085ΔgC-R (p=0.0001, p=0.0001 and p=0.001 at 48h; p=0.011, p=0.010 and p=0.011 at 72h; compared to 2085). The viral titers determined for v2085ΔgC, v2085-GFPΔgC and v2085-H5 were not significantly different from each other (p=0.954, 0.999 and 0.958 at 48h; p=0.989, 0.994 and 0.995 at 72h). Although v2085ΔgC-R did not quite reach the titers of the parental 2085 virus, the differences between the two viruses proved to not be significantly different (p=0.059 at 48h and 0.107 at 72h) (Fig 2.6B).

Finally, virus titers were determined after trypsinization of virus-infected CEC and plating on fresh, uninfected cells directly without freeze-thawing as we had observed significant sensitivity of DEV to freeze-thawing in our initial experiments. The viral titers determined by this method again confirmed similar growth properties for parental 2085 virus and v2085ΔgC-R (p=0.611 at 48h and p=0.158 at 72h). The gC deletion viruses v2085ΔgC, v2085-GFPΔgC, and v2085-H5 exhibited a slight reduction when compared to the parental and revertant viruses that ranged from 2- to 7-fold at 48h and 20- to 50-fold at 72h. However, the titers were not significantly different from parental 2085 virus at either time point, however (p=0.128, p=0.113 and p=0.143 at 48h; p=0.117, p=0.124 and p=0.127 at 72h)(Fig 2.6C).

Taken together, our results indicated that deletion of gC in DEV does not markedly affect the production of infectious progeny virus and that cell-to-cell spread of DEV appears to not be dependent on gC. Based on the observation that gC-negative DEV induce larger plaque sizes, possibly caused by the fact that (soluble) gC can no longer be produced by infected cells and interfere with infection efficiencies by binding to the receptor, as well as the significantly reduced viral titers in infected-cell supernatants and after freeze-thawing, we conclude that gC is involved in both virus entry and egress. Such a dual function in virus replication has been described before for a number of related viruses (Adamiak et al., 2010; Azab et al., 2010; Jarosinski and Osterrieder, 2010; Osterrieder, 1999; Rue and Ryan, 2008; Scheper et al., 2010; Tischer et al., 2005; Trybala et al., 1993).

2.5. Conclusions

Taken together, our study shows that an infectious BAC clone of DEV could be established. The infectious clone provides an efficient tool that allows straightforward manipulation using bacterial recombination systems. Utilizing the E. coli recombination machinery, we were able to delete one of the major structural DEV proteins, gC. We were also able to show that gC plays an important role in viral egress. We also tested the potential of DEV for its vector potential, similar to what has been published for other herpesviruses such as EHV-1, HSV-1, MDV or herpesvirus of turkeys (HVT), DEV has promise to be used in protecting against other (viral) diseases and would represent the first modified live virus vector in ducks. Successful construction of DEV expressing H5 of a highly pathogenic AIV demonstrated that such use of DEV as a vector is possible as H5 was expressed at high levels and reasonable virus titers were reached although a foreign protein was produced. Lastly, the cloning of entire DEV genomes allows analysis of further genes involved in virus growth or pathogenesis as well the ready determination of the nucleic acid sequence of a virulent DEV.
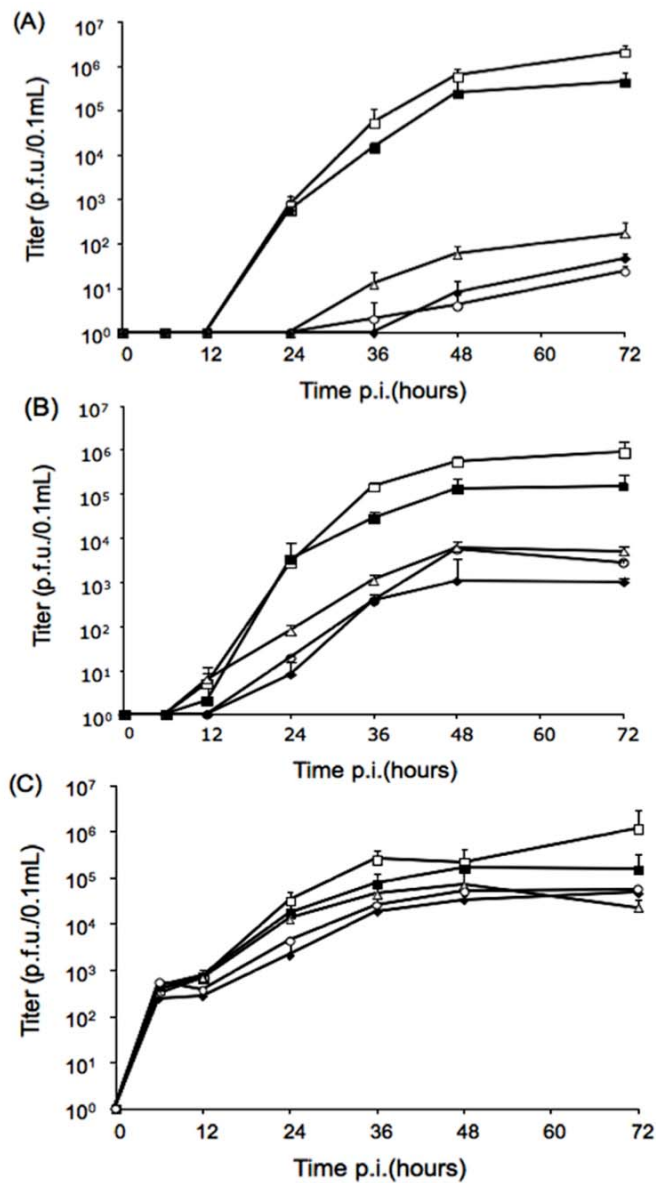
Figure 2.6. Multi-step growth kinetics of 2085, v2085ΔgC-R, v2085ΔgC, v2085-GFPΔgC and v2085_H5 on CEC.

Titers of 2085 (□), v2085ΔgC-R (■), v2085ΔgC (△), v2085-GFPΔgC (○) and v2085_H5 (◆) of infected-cell supernatants (A), cell-associated virus (B) and virus in trypsinized cells (C) are shown for the indicated time points after infection with an MOI of 0.01. Titers are given as plaque forming units in 0.1 mL. Shown are means of virus titers as determined by three independent experiments, standard deviations are shown with the error bars.

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Chapter 3. Complete Genome Sequence of Virulent Duck Enteritis Virus Strain 2085 and Comparison with Genome Sequences of Virulent and Attenuated DEV Strains

3.1. Summary

We here report the complete genome sequence of the duck enteritis virus (DEV) wild-type strain 2085, an avian herpesvirus (GenBank ID: JF999965). The nucleotide sequence was derived from the 2085 genome cloned as an infectious bacterial artificial chromosome (BAC) clone. The DEV 2085 genome is 160,649 base pair (-bp) in length and encodes 78 predicted open reading frames (ORFs), a number identical to that identified for the attenuated DEV VAC strain (GenBank ID: EU082088.2) Comparison of the genome sequences DEV 2085 and VAC with partial sequences of the virulent CHv strain and the attenuated strain Clone-03 was carried out to identify nucleotide or amino acid polymorphisms that potentially contribute to DEV virulence. No amino acid changes were identified in 24 of the 78 ORFs, a result indicating high conservation in DEV independently of strain origin or virulence. In addition, 39 ORFs contain non-synonymous nucleotide substitutions, while 15 ORFs had nucleotide insertions or deletions, frame-shift mutations and/or non-synonymous nucleotide substitutions with an effect on ORF initiation or termination. In 7 of the 15 ORFs with high and 27 of the 39 ORFs with low variability, polymorphisms were exclusively found in DEV 2085, a finding that likely is a result of a different origin of 2085 (Europe) or VAC, Clone-03 and CHv (Eastern Asia). Five ORFs (UL2, UL12, US10, UL47 and UL41) with polymorphisms were identical between the virulent DEV 2085 and CHv but different from VAC or Clone-03. They, individually or in combination, represent DEV virulence factors. Our comparative analysis of four DEV sequences provides a comprehensive overview of DEV genome structure and identifies ORFs that are changed during serial virus passage.

3.2. Introduction

Duck plague or duck viral enteritis (Willemse et al.) is an acute, infectious and often lethal disease of ducks, geese, swans and many other species of waterfowl within the order *Anseriformes*. The disease occurs world-wide and is caused by duck enteritis virus (DEV). Infection of domesticated ducks can be accompanied with high mortality, carcass condemnation and reduction of egg production or hatchability, resulting in losses for the waterfowl breeding industry (Janson, 1968; Leibovitz, 1968; Leibovitz and Hwang, 1968; Walker et al., 1969). In addition, DEV causes variable mortality in wild water-fowl, where the disease incidence and

disease severity vary between species and epizootics (Kaleta et al., 2007; Keymer and Gough, 1986). Upon primary infection, DEV establishes latency in the trigeminal ganglia (TG) from which the virus can reactivate leading to further disease outbreaks (Shawky and Schat, 2002). The most significant gross lesions include caseous plaques, necrotic bands and hemorrhage on digestive mucosal tissues (Converse and Kidd, 2001; Gough and Alexander, 1990; Kaleta et al., 2007; Leibovitz and Hwang, 1968; Montgomery et al., 1981; Wobeser, 1987).

DEV is taxonomically classified as *Anatid herpesvirus 1* in the *Alphaherpesvirinae* subfamily, but not assigned to any genus according to the latest report of the International Committee on Taxonomy of Viruses (ICTV) released in 2009. Virulence varies for different DEV isolates. Attenuated or naturally apathogenic DEV strains are used as live vaccines that can provide efficient protection under industry settings against clinical duck plague (Hess and Dardiri, 1968; Janson, 1968; Li et al., 2009; Lian et al., 2010; Lin et al., 1984a; Lin et al., 1984b; Liu et al., 2007; Spieker, 1977).

In recent years, sequences of a number of individual open reading frames (ORFs) of different DEV strains have been published. The first complete genome sequence of DEV strain VAC, a Chinese vaccine strain attenuated by serial passage on chicken embryo cells (CEC), was published in 2009 (Li et al., 2009). Amongst the other published sequences were almost all ORFs of Clone-03 that was derived from the attenuated VAC strain (Liu et al., 2009) and CHv, a virulent strain isolated in China (Lian et al., 2010). Analysis of the sequenced DEV VAC genome showed that it represents a type D herpesvirus genome (Li et al., 2009).

While molecular characterization of individual DEV proteins such as those encoded by UL15, UL51, UL45 or UL24 has advanced over the years (Liu et al., 2009; Shen et al., 2010; Xiang et al., 2011; Zhu et al., 2011), little is known of the determinants of DEV pathogenicity. We therefore reasoned that determination of the complete genome sequence of a virulent DEV strain would allow comprehensive comparison of genomes of virulent and apathogenic strains and identification of candidate genes associated with DEV virulence. We therefore determined the complete genome sequence of the virulent wild-type DEV strain 2085. To facilitate sequence analysis and to generate a tool for genetic manipulation of the virus genomes, an infectious clone from the 2085 DEV strain (p2085) was generated in our laboratory (Wang and Osterrieder, 2011). Based on our sequencing data, we compared the 2085 genomic sequence with that of the attenuated VAC strain and the published ORFs of Clone-03 and CHv. The comparison allowed us to identify possible virulence factors and strain-specific sequences that

will be useful for diagnostic differentiation between strains of different pathogenic potential and geographic provenance.

3.3. Materials and Methods

3.3.1. Virus, cells and BAC

DEV strain 2085 (kindly provided by Dr. Erhard Kaleta, Justus-Liebig-Universität Gießen) was isolated from an outbreak of duck plague in Germany in 2005 (Kaleta et al., 2007). The 2085 was isolated on CECs and propagated as described before (Li et al., 2009). Generation of an infectious BAC clone of 2085 (p2085) was achieved by insertion of a mini-F vector into the Glycoprotein C (gC) locus of DEV strain 2085 (10th passage on CEC), thereby deleting the ORF. Characterization of the infectious clone and of BAC-derived virus as well as a gC revertant virus was described earlier (Wang and Osterrieder, 2011). The p2085 clone was maintained in *Escherichia coli* GS1783 cells (Tischer et al., 2010).

3.3.2. DNA isolation and genome sequencing

p2085 Deoxyribonucleic acid (DNA) was isolated by column affinity purification (Midi-prep kit, Qiagen). A DNA library for sequencing with Genome Sequencer FLX (Roche) was prepared as described earlier (Wiley et al., 2009). The double-stranded DNA (dsDNA) library was bound to library capture beads and a single-stranded template DNA (ssDNA) library was generated. The ssDNA library was prepared for sequencing and sequenced according to the manufacturer's instructions. Raw sequence data were assembled using the GS assembler software Newbler (v. 2.3; Roche). After p2085 genome assembly, the sequence of the inserted mini-F was replaced with the deleted UL44 (gC) gene that was sequenced separately to obtain a complete genome sequence of DEV strain 2085. All identified nucleotide insertion/deletion mutations or frame-shift mutations relative to published sequences were verified by Sanger sequencing using specific primers and p2085 DNA and DNA obtained from CEC infected with parental 2085 virus (StarSEQ).

3.3.3 DNA sequence analysis

The complete genome of 2085 was annotated through the GATU online annotation software provided by Viral Bioinformatics Resource Center (http://athena.bioc.uvic.ca/) using the DEV VAC complete sequence as a reference (GenBank ID: EU082088.2). The published ORFs of Clone-03 and CHv were compared with those of 2085 or VAC using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence comparisons of ORFs between all four strains were performed using VectorNTI (Invitrogen) and edited manually. For ORFs that exhibited

mutations at predicted initiation or stop sites and frame-shift mutations, additional sequences up- or downstream of genes were also assembled. Non-synonymous substitutions were listed individually and the nucleotide sequences of ORFs containing nucleotide insertions, deletions or frame-shift mutations were further scrutinized.

3.4. Results and Discussion

3.4.1. Genomic organization of DEV strain 2085

The length of the circular p2085 genome without the BAC vector sequences and after insertion of the UL44 (gC) gene sequenced separately was determined at 160,649-bp (GenBank ID: JF999965). The length of the DEV 2085 genome exceeds that of VAC DEV by 2,558-bp (Li et al., 2009)(Table 3.1). There is colinearity of 98% of the VAC genome with the 2085 genome when using NCBI BLAST, but the orientation of the unique-short (US) segment within the DEV 2085 sequence cloned as a BAC (p2085) is reverse to that of DEV strain VAC, likely due to inversion of the segment, a feature that is common among type D herpesvirus genomes. We were able to identify the 78 annotated ORFs of the attenuated VAC strain in the virulent 2085 strain and no additional ORFs were observed. The 2085 Guanine-cytosine content (G+C content) is 44.93%, and virtually identical to that of the VAC strain.

Table 3.1. Complete genome Comparison of DEV strains 2085 and VAC

Strain	UL	US	IRS/TRS	Total length
2085	122,141	12,724	12,892	160,649
VAC	119,306	12,727	13,029	158,091

The unique-long segment (UL) extends from nt position 1 to 122,141 and contains an additional 2,835-bp when compared with strain VAC. The observed difference can mainly be attributed to differences in LORF11, UL2 and LORF3, which specify 2343, 441 and 33 additional nucleotides (nt), respectively, when compared to strain VAC (Supplementary table 1). The US extends from nt 135,034 to nt 147,757 and is shorter by only 3-bp compared to strain VAC, but, as mentioned earlier, is present in an inverse orientation relative to the UL segment when compared to the VAC DEV strain. Similar isomerizations have been demonstrated in the case of equine herpesvirus 1, bovine herpesvirus 1, equine herpesvirus 3, pseudorabies virus and varicella-zoster virus (Campagnolo et al., 2001; Chowdhury et al., 1990; Davison et al., 1993; Liu et al., 2010; Shawky and Schat, 2002; Spatz et al., 2011). The internal and terminal inverted repeat sequences (IRS/TRS) that flank the US are 137-bp shorter than that of strain VAC for each repeat. The missing sequences include the UL-IRS junction where 47-bp are missing and the IRS-US junction with a deletion of 90-bp. Both deletions at the junction between the IRS and the unique regions are in non-coding regions.

3.4.2. Comparison of ORFs between DEV strains 2085, VAC, Clone-03 and CHv.

Along with the complete genome sequence of 2085 and VAC, almost complete sets of sequences for ORFs of DEV strains Clone-03 and CHv are found in GenBank. The only genes missing using NCBI BLAST are UL44 and UL32 of Clone-03, and UL44.5, UL23, UL17.5 and UL15b of strain CHv. In addition, the deposited CHv LORF11 sequence (723-bp, GenBank accession number FJ232031.1) exhibits 100% identity to the carboxyterminal part of 2085 and we concluded that the CHv LORF11 sequence represents a partial sequence.

All open reading frames available were compared through NCBI BLAST or VectorNTI to identify the nucleotide (Supplementary table 1) and amino acid polymorphisms (Supplementary table 2). There are 5 genes (UL2, UL10, UL13, UL53 and US10), which initiate at different start codons in the 4 strains, but no other polymorphisms were found that would cause the differences in ORF initiation. We surmise that the differences are caused by different methods employed for the prediction of the genes in question (Liu et al., 2008; Zhang et al., 2010), while an alternative explanation is that both initiation sites can be utilized similar to the situation determined for the UL36 and UL36.5 genes. We therefore simplified the comparison of the ORFs by modifications according to the predictions made in the case of VAC or 2085 (Li et al., 2009), even though the published data was still kept without modification.

There were 8 genes (UL7, UL11, UL16, UL26.5, UL30, UL44, UL45 and UL46) in the UL and four genes (two repeats of US1, US6 and US3) in the US region that exhibited 100% identity on the nucleotide level between all strains (Supplementary table 1). Eleven genes (UL8, UL14, UL24, UL32, UL33, UL35, UL38, UL49.5, UL50, UL51 and UL53) in the UL and one gene (US2) in the US segment were 100% identical on the amino acid level between all four strains and hence only synonymous nucleotide substitutions were identified. In summary, a total of 24 genes exhibited high levels of similarity and conservation among the four DEV strains analyzed, indicating that these genes likely do not play a role in the attenuation process of the Clone-03 and VAC DEV strains. Similarly, the 24 identical genes seem to be stable beyond geographic boundaries as European (2085) and Asian strains specify identical or nearly identical nucleotide sequences.

When we stratified the 24 highly conserved genes according to functions, we found 4 glycoprotein genes (gK, gN, gC and gD – UL44, UL49.5, UL53 and US6) of the 12 glycoproteins encoded by DEV. Nine additional structural protein genes (UL11, UL14, UL16, UL26.5, UL35, UL38, UL45, UL46 and UL51) are also included in this group of highly conserved genes. The remainder of highly conserved and annotated genes encode proteins involved in DNA replication

or nucleotide metabolism include UL8, UL16, UL32, UL33, UL50, US1 and US3. Finally, 3 genes with unknown function are also part of this group and include UL7, UL24 and US2.

The gene products involved in viral DNA metabolism and structural proteins are known to be highly conserved within the entire subfamily of the *Alphaherpesvirinae* (Ghanem et al., 2007; Li et al., 2009; Subak-Sharpe and Dargan, 1998). Glycoproteins play important roles in virus entry and egress and contribute to host range and variability (Adamiak et al., 2010; Jarosinski et al., 2010; Li et al., 2009; Osterrieder, 1999; Scheper et al., 2010; Spatz and Schat, 2011) . Given that the VAC and Clone-03 strains are highly adapted to CECs, while 2085 and CHv are virulent strains that underwent very limited cell culture adaptation on CEC (2085) or duck embryo cells (CHv), our results indicate that 4 DEV glycoproteins (gC, gD, gK and gN) seem to play no or only a minor role for DEV host range and virulence, at least with respect to the four strains studied here.

UL37 (tegument protein), UL34 (nuclear egress protein; primary virion envelope protein; tail-anchored type II nuclear membrane protein), UL12 (DNA recombinase/alkaline exonuclease) and US7. Clone-03 has 4 strain-specific polymorphisms in LORF11, UL55 (virion assembly and maturation), UL12 and UL9 (origin-binding protein). In contrast, we were unable to identify strain-specific modifications of CHv in the 15 highly variable ORFs. Three of the polymorphisms are shared by the virulent 2085 and CHv strains and concern the UL2 (Uracil-DNA glycosylase), UL12 and US10 (virion protein) genes. The rather massive changes in UL2 and UL12 of CHv and 2085 relative to the avirulent VAC and Clone-03 DEV strains may indicate that those changes play a major role in the loss of virulence of the modified live vaccine strains.

3.4.2.1. Variations in LORF11

The 4 ORFs with nucleotide deletions and insertions are LORF3, UL2, UL9 and LORF11, all of which are located in UL region (Fig 3.1). LORF11 has orthologues only in other avian alphaherpesviruses, namely Marek's disease virus (MDV), gallid herpesvirus 3 (GaHV-3), meleagrid herpesvirus (herpesvirus of turkeys, HVT) and infectious laryngotracheitis virus (ILTV). It was shown that MDV LORF11 plays a role in virus replication and virulence, but no further information on its function is available (Lee et al., 2007). The comparison between LORF11 sequences showed a high degree of variation between 2085, VAC and Clone-03. All three strains share a 164 amino acid domain at the aminoterminus and a 60 amino acid domain at the carboxyterminus; however, the VAC strain lacks a 532 amino acid domain that is shared by strains 2085 and Clone-03, whereas strain 2085 does not have a stretch of 47 amino acids downstream of the aminoterminal 164 amino acid domain, which is shared by VAC and Clone-

03. In addition, the VAC strain has a strain-specific 4 amino acid insertion, while strain 2085 has a strain-specific 300 amino acid insertion (Fig 3.1). Unfortunately, the whole CHv LORF11 sequence is not available, but it is apparent from the partial sequence that it is clearly different from VAC. While the nucleotide sequences of LORF11 of the original VAC and Clone-03 strains before serial passage on CECs were not available either, we concluded that the 2085-specific 900-bp insertion (300 amino acids) is specific for European strains and that the VAC and Clone-03 specific 142-bp nucleotide stretch (47 amino acids) is specific for Asian strains. An alternative explanation is that the 142-bp insertion is a result of serial passage of DEV on CECs and an adaptive mutation that facilitates growth in chicken cells. Whether the 901-bp insertion in LORF11 of 2085 is related to virulence is unclear at present, but will be studied by generation of deletion mutants of 2085. An attenuating effect of LORF11 mutations were shown in the case of MDV, where deletion of LORF11 did not affect virus growth in vitro but significantly affected replication in vivo and resulted in attenuation (Lee et al., 2007).

3.4.2.2. Variations in UL2

UL2 is predicted to encode an enzyme important for viral replication, uracil DNA glycosylase, the homolog of which was shown to play a prominent role in HSV-1 DNA replication (Bogani et al., 2009; Pyles and Thompson, 1994). The published DEV UL2 sequences showed different initiation sites between the 2085 and CHv on the one hand, and VAC and Clone-03 on the other hand (Fig 3.1). However, the sequences upstream of the 2085 and CHv start codon were identical to those reported for the vaccine strains and we hence concluded that the differences are caused by differences in the ORF-predicting software programs. When we further compared the UL2 sequences of the 4 DEV strains, we could, however, identify an extra 528-bp stretch in the UL2 sequences of 2085 and CHv. Because 2085 and CHv are virulent strains and were not extensively passaged, the additional 176 amino acid stretch may contribute to UL2 protein activity. At the same time it remains unknown whether loss of the 176 amino acid sequence stretch is a result of continuous virus passage in CECs. Another polymorphism of 3 nucleotide deletions and 1 non-synonymous substitution in the 277-bp/280-bp stretch at the carboxyterminus is identical between 2085, CHv and Clone-03, but not VAC. It is difficult to interpret the available data due to the fact that Clone-03 is a virus clone derived from the VAC population (Liu et al., 2010; Liu et al., 2009). It is clear, however, that the insertions, deletions and polymorphisms regarding the 277-bp/ 280-bp stretch resulting in an 18 amino acid substitution to the primary protein sequence does seem not critical for virulence or growth ability on CEC.

3.4.2.3. Variations in UL9

DEV UL9 is the homolog of the HSV-1 origin-binding helicase (Olsson et al., 2009). Even though there are several nucleotide polymorphisms in the UL9 sequences of 2085, CHv and VAC, the protein sequences are 100% identical. However, the UL9 coding sequences of Clone-03 contain two 74-bp nt repeats instead of one found in the other three strains. The second repeat in Clone-03 UL9 causes a frame-shift that results in a truncated protein of 766 amino acids instead of a 859 amino acid polypeptide (Fig 3.1). This result indicates that the polymorphism may be a determinant of virulence but that a significant truncation of the UL9 protein is still capable of supporting virus growth in vitro and in vivo. The mutation was not observed in published UL9 sequences of 6 HSV-1 strains or 13 MDV strains when compared by NCBI Blast (http://blast.ncbi.nlm.nih.gov/ Blast.cgi), a result indicating that this repeat polymorphism is specific to DEV.

3.4.2.4. Variations in LORF3

The last ORF with sequence variations is LORF3, whose function is still unknown and for which orthologues were only identified in the avian alphaherpesviruses MDV, GaHV-3, HVT, ILTV and psittacine herpesvirus (Li et al., 2009). The 2085 strain lacks one copy of an 11 amino acid repeat, which is present as two copies in the case of the LORF3 protein of Clone-03, VAC and CHv, a situation reminiscent of the UL9 gene (Fig 3.1). There are 2 non-synonymous amino acid substitutions (Ala to Thr and Asp to Glu at position 25 and position 317/328, respectively) in strain 2085 (Supplementary table 2). Possible explanations are that the variable number of repeated sequences and the polymorphisms are a consequence of virus propagation in cultured cells or may indeed point to geographic differences between European (2085) and Asian DEV isolates (VAC, Clone-3, CHv).

3.4.2.5. Variations in other ORFs present in the unique-long genome segment

There are 8 additional UL genes that show frame-shift mutations, namely UL6, UL12,, UL13, UL34, UL37, UL48, UL49 and UL55 (Supplementary table 1 and 2; Fig 3.2 and 3.3). The coding region of Clone-3 UL55 was altered by a frame-shift mutation by adding an extra adenosine at position121, but the ORF is restored by a compensatory frame–shift caused by deletion of a thymidine at position 174. The 2 mutations cause a difference in 17 codons, along with a non-synonymous substitution of Ala of Clone-03 from Val present in the other strains at position 93,
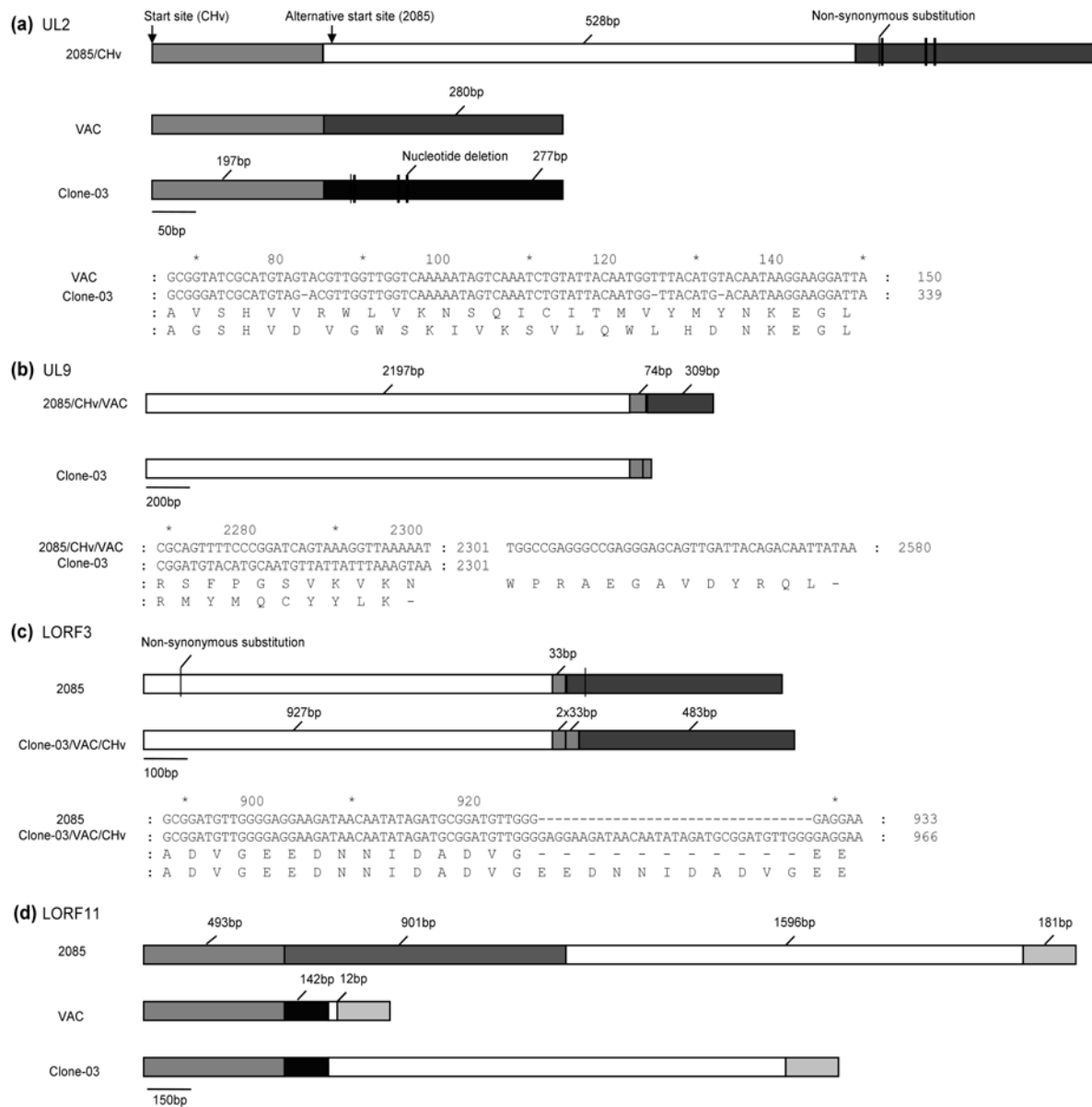
Figure 3.1. Comparison of UL2, UL9, UL12, LORF3 and LORF11 in various DEV strains.
Deletions and insertions in UL2 (a), UL9 (b), LORF3 (c) and LORF11 (d) are shown for the indicated DEV strains. Regardless of the orientation in the virus sequence, all ORFs are shown with the start codon on the left and the stop codon on the right hand side. Lengths and sites of nucleotides in ORFs are marked. Abbreviations of amino acids are given. Rectangles with the same type of shade indicate identical sequences. Arrows show the start codon predictions according to published sequences in GenBank. Initiation codons are underlined. Non-synonymous nucleotide changes are shown by vertical bars, non-synonymous changes as well as deletions and insertions are indicated.

(a) UL6

15bp — Non-synonymous substitution — 2374bp

2085

Nucleotide deletion — 2373bp

VAC/Clone-03/CHv

100bp

```
                    *        20         *        40         *        60         *        80
2085           : ATGTCAGCTAACCAATGCGGGATATGTCCCGCCCAGCACGACTGGGGTACTGGGAGGAGCAAACAAAGACGTATCAATACCGGC :  84
VAC/Clone-03/CHv :       ATGCGGGATATGTCCCGCCCAGCACGACTG-GGTACTGGGAGGAGCAAACAAAGACGTATCAATACCGGC :  69
               : M  S  A  N  Q  C  G  I  C  P  A  Q  H  D  W  G  T  G  R  S  K  Q  R  R  I  N  T  G
                 M  R  D  M  S  R  P  A  R  L     G  T  G  R  S  K  Q  R  R  I  N  T  G
```

(b) UL12

236bp — 1104bp — Nucleotide deletion — 349bp

2085/CHv

1103bp

VAC

1105bp

Clone-03

100bp

```
                 *       20          *       240                         *       1340
2085/CHv : ATGAGTGGGGATATAGATGGT :  21   CTACCACAATGTATTCATGAG :  249   CCC-AAACATTCCAACTTTAAACA : 1343
VAC      :                             ATGTAT-CATGAG :  12   CCC-AAACATTCCAACTTTAAACA : 1106
Clone-03 : ATGAGTGGGGATATAGATGGT :  21   CTACCACAATGTATTCATGAG :  249   CCCCAAACATTCCAACTTTAA    : 1341
         : M  S  G  D  I  D  G        L  P  Q  C  I  H  E         P  K  H  S  N  F  K
         :                            M  Y     H  E              P  K  H  S  N  F  K
         : M  S  G  D  I  D  G        L  P  Q  C  I  H  E         P  Q  T  F  Q  L  -

2085/CHv : GCCTTCGATACGACAGCTACACAACTATGGGACAAGCTCAATCCTACGGGAGCGGTGGATGGTGCTGCAGAAGAAACATATTAA : 1689
VAC      : GCCTTCGATACGACAGCTACACAACTATGGGACAAGCTCAATCCTACGGGAGCGGTGGATGGTGCTGCAGAAGAAACATATTAA : 1452
         : A  F  D  T  T  A  T  Q  L  W  D  K  L  N  P  T  G  A  V  D  G  A  A  E  E  T  Y  -
         : A  F  D  T  T  A  T  Q  L  W  D  K  L  N  P  T  G  A  V  D  G  A  A  E  E  T  Y  -
```

(c) UL13

Start site (Clone-03/CHv) Alternative start (VAC) — 1431bp

VAC/Clone-03/CHv

144bp

2085

100bp

```
                                     *        20         100        *
VAC          :                     ATGAATCCTATCGGAAGCTCC :  21   GAAAATTCAATCGAAATGCGCGAC : 117
Clone-03/CHv : ATGGCTGGACGAAGACGACGA :  21   ATGAATCCTATCGGAAGCTCC :  57   GAAAATTCAATCGAAATGCGCGAC : 153
2085         :                                                           ATGCGCGAC :   9
             :                        M  N  P  I  G  S  S         E  N  S  I  E  M  R  D
             : M  A  G  R  R  R  R    M  N  P  I  G  S  S         E  N  S  I  E  M  R  D
             :                                                           M  R  D
```

Figure 3.2. Alignments of nucleotide and amino acid sequences of ORFs in the UL region with frame-shift mutations in start codons.

Shown are UL6 (a), UL12 (b) and UL13(c). of DEV strain 2085, VAC, Clone-03 and CHv. Regardless of the orientation in the virus sequence, all ORFs are shown with the start codon on the left and the stop codon on the right hand side.Lengths and sites of nucleotides in ORFs are marked. Abbreviations of amino acids are given. Rectangles with the same type of shading in indicate identical sequences. Non-synonymous nucleotide changes are shown by vertical bars, non-synonymous changes as well as deletions and insertions are indicated. Scale bar is 100-bp.

(a) UL34

677bp   One nucleotide deletion   153bp
2085/Clone-03/CHv

678bp   Non-synonymous substitution
VAC

50bp

```
                    *        660        *        680        790        *        810        *        830
2085/Clone-03/CHv : CCTTTAGAAGATTTGGACCCAAACAGT-AACGA : 680   ATGTTTGCGTTAGTCATTTGGCCTTTTGGTGGCCGTATCTAG : 831
VAC               : CCTTTAGAAGATTTGGACCCAAACAGTTAA    : 678
                  : P  L  E  D  L  D  P  N  S  N               M  F  A  L  V  I  W  P  F  G  G  R  I  -
                  : P  L  E  D  L  D  P  N  S  -
```

(b) UL37

Non-synonymous substitution   3201bp
2085

3201bp
Clone-03/CHv

Nucleotide deletion   3198bp
VAC

200bp

```
                    580        *        600        *        620        *        640        *        660
2085/Clone-03/CHv : TTACTCGACTCAGAAACGTCTATGGATTCCGAAAAGATCGTGAACCCTTTACTAGCCGATACATACGCATTAATATATGATTTT : 660
VAC               : TTACTCGACTCAGAA-CGTCT-TGGATTCCGAAAAGATCGTGTACCCTTTACCAGCCGATACATACGCATTA-TATATGATTTT : 657
                  : L  L  D  S  E  T  S  M  D  S  E  K  I  V  N  P  L  L  A  D  T  Y  A  L  I  Y  D  F
                  : L  L  D  S  E  R  L  G  F  R  K  D  R  V  P  F  T  S  R  Y  I  R  I  I  Y  D  F
```

(c) UL49

740bp   One nucleotide deletion
VAC/Clone-03/CHv

22bp

741bp
2085

50bp

```
                    *        700        *        720        *        740        *        760
VAC/Clone-03/CHv : TCCGTTGCCCCGGGGGGAAAA-GACGCACAGCATGCAAGGGCTCCGTCCAGCCACCGTAACAGTTCAAGAAGACCACAATAA : 762
2085             : TCCGTTGCCCCGGGGGGAAAAAGACGCACAGCATGCAAGGGCTCCGTCCAGCCACCGTAA                        : 741
                 : S  V  A  P  G  G  K  D  A  Q  H  A  R  A  P  S  S  H  R  N  S  S  R  R  P  Q  -
                 : S  V  A  P  G  G  K  R  R  T  A  C  K  G  S  V  Q  P  P  -
```

(d) UL55

Nucleotide deletion   Non-synonymous substitution
2085/VAC/CHv

Nucleotide deletion
Clone-03

50bp

```
                    *        120        *        140        *        160        *        180
2085/VAC/CHv : ATGTTTTACATGCCCAAGAAG-AAGGACCACTGTGTTACACCAAGACCAGAGGTTGTTCTAGAGAGCGCGTCGTTTTTTGAACAG : 183
Clone-03     : ATGTTTTACATGCCCAAGAAGAAAGGACCACTGTGTTACACCAAGACCAGAGGTTGTTCTAGAGAGCGCGTCGTT-TTTGAACAG : 183
             : M  F  Y  M  P  K  K  K  D  H  C  V  T  P  R  P  E  V  V  L  E  S  A  S  F  F  E  Q
             : M  F  Y  M  P  K  K  G  P  L  C  Y  T  K  T  R  G  C  S  R  E  R  V  V  F  E  Q
```

Figure 3.3. Alignments of nucleotide and amino acid sequence of ORFs in the UL segment containing premature stop sites or frame-shift mutations in the coding region.

The UL34 (a), UL37 (b), UL49 (c) and UL55 (d) genes are shown. Regardless of the orientation in the virus sequence, all ORFs are shown with the start codon on the left and the stop codon on the right hand side. Lengths and sites of nucleotides in ORFs are marked. Abbreviations of amino acids are given. Rectangles with the same type of shading in indicate identical sequences. Arrows show the start codon predictions according to published sequences in GenBank. Initiation codons are underlined.
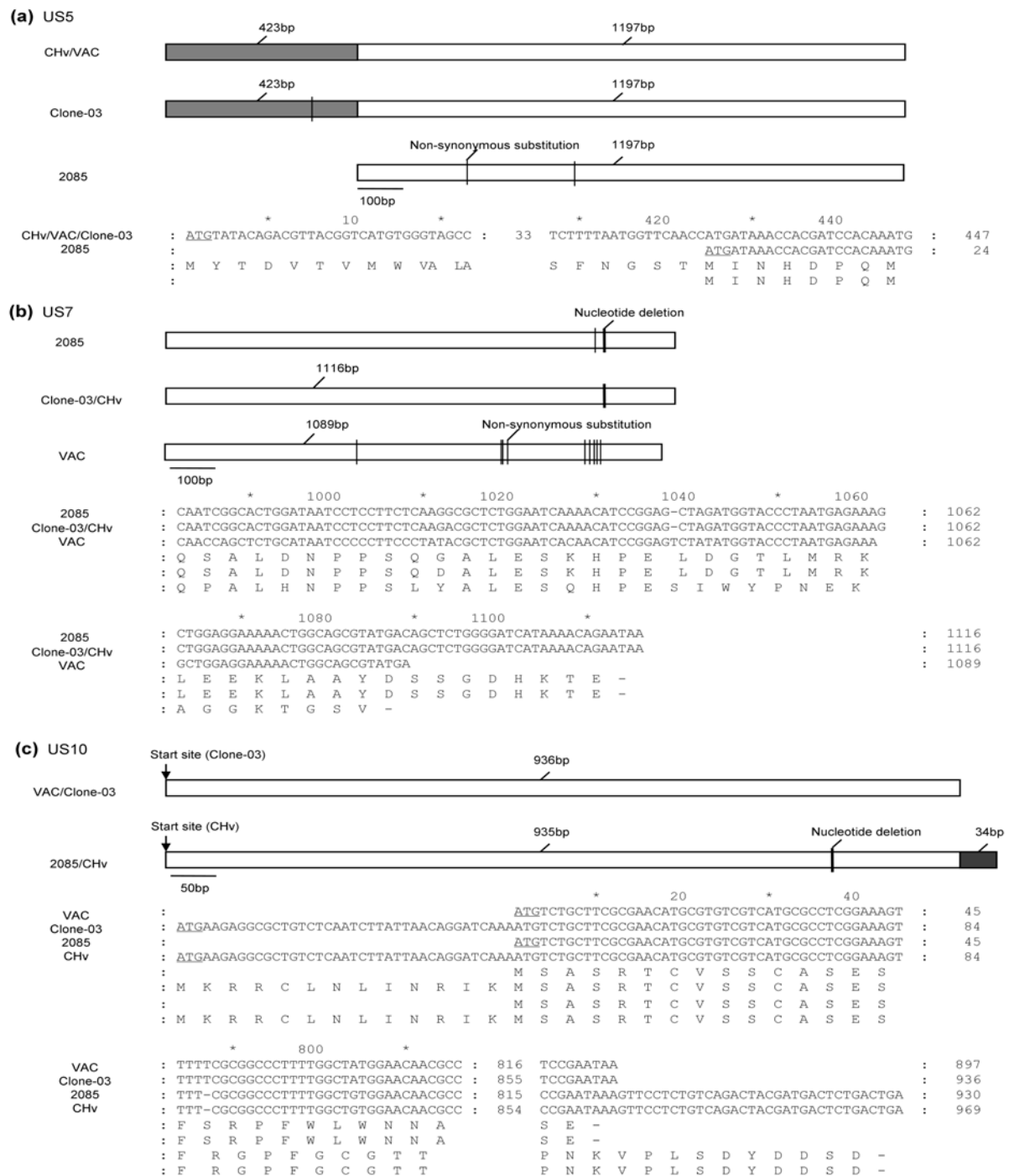
Figure 3.4. Alignments of nucleotide and amino acid sequence of ORFs in the US segment containing frame-shift mutations in the coding regions.

Shown are US5 (a), US7 (b) and US10 (c) ORFs. Regardless of the orientation in the virus sequence, all ORFs are shown with the start codon on the left and the stop codon on the right hand side. Lengths and sites of nucleotides in ORFs are marked. Abbreviations of amino acids are given. Rectangles with the same type of shading in indicate identical sequences. Arrows show the start codon predictions. Initiation codons are underlined. Scale bars are 100bp and 50bp, respectively.

indicating a possible attenuating mutation of Clone-03. Similarly, an extra adenosine base at nt position 703 of 2085 UL49 causes a truncation of the coding sequence of 21 nt and results in a 246 amino acid protein compared to UL49 proteins of 253 amino acids in length for the other DEV isolates, along with the substitution of 12 amino acids at the carboxyterminus. A substitution of adenosine with guanosine in UL48 of strain 2085 in the start codon results in a frame-shift and translation initiation 6 nucleotides downstream.In the case of UL37, besides two non-synonymous substitutions of Gly and Asn in strain 2085 at position 5 and 385, respectively, and one non-synonymous substitution of Ile of VAC at position 170, there are 3 nucleotides missing in the VAC gene (adenosine at pos. 592, thymidine at pos. 597 and adenosine at pos. 648), which result in an alteration of a 19 amino acid stretch at position 198 – 216 of the 1066 amino acid protein. The polymorphisms in VAC UL37 could indicate these changes represent attenuating mutations; however, no specific polymorphisms were observed for Clone-03, which, as outlined earlier, was derived from strain VAC and also is attenuated.

Except for a non-synonymous substitution (Leu to Ser at position 124 of strain CHv), an extra thymidine at nucleotide position 676 of VAC UL34 causes a stop codon that in turn would result in the expression of a truncated protein of 225 amino acids, hence, a protein considerably shorter (51 amino acids) than those encoded by the other strains. The product of UL34 homologues of herpesviruses is an essential protein that plays an important role in nuclear egress (Neubauer et al., 2002; Schnee et al., 2006; Wills et al., 2009). Truncation of a 51 amino acid stretch of VAC UL34 maybe therefore represent a mutation that contributes to the attenuation of this strain. Again, however, this truncation is absent in the clonal derivative of VAC, Clone-03 making a firm interpretation of this polymorphism virtually impossible.

For UL13 of 2085, an alteration of guanosine to thymidine at position 13 causes the codon being changed from GGA to a stop codon (TGA), resulting in the use of an alternative start codon that leads to a truncation of 36 amino acids at the extreme aminoterminus. The product of UL13 of alphaherpesvirus is a protein kinase (Asai et al., 2007) that is required for horizontal transmission of MDV (Jarosinski and Osterrieder, 2010). The mutation identified in the case of DEV 2085 may serve as a geographic marker of European isolates, but certainly does not affect virus growth in vitro or virulence in vivo. We were not able to identify by alignments with other alphaherpesviral UL13 protein predictions that the 36 amino acids at the aminoterminus present in the Asian strains represent a conserved domain. We would, therefore, predict that, despite the truncation, the 2085 UL13 protein is capable of exerting all the functions ascribed to the serine/protein kinase.

With regard to the UL12 proteins of VAC and Clone-03, relatively significant mutations were identified in the two vaccine strains (Fig 3.2). We noted truncations at the amino- and carboxytermini when compared to 2085 or CHv, which have identical UL12 genes. Deletion of a thymidine at nt position 243 causes shift of the initiation site of VAC UL12 237-bp downstream, which results in the absence of the aminoterminal 79 amino acids, which are replaced with 2 different residues. An extra cytosine at position 1324 leads to a carboxyterminal truncation of the Clone-03 UL12 product by 116 amino acids that are replaced with 5 non-synonymous amino acids. This result indicates that UL12 of DEV may play an important role for the virulence of 2085 and CHv, and that truncation at either the amino- or carboxyterminus may be one of the major candidates that results in DEV attenuation without affecting virus growth in vitro or vivo.

With regard to the UL6 ORF, we noted a difference between the 2085 and the remaining isolates. An extra guanosine at position 45 in the 2085 UL6 sequence causes the initiation site to be altered, resulting in 5 additional amino acids and a difference of 10 amino acids at the aminoterminus. It was shown that the HSV-1 UL6 product is required for portal ring formation, a structure that participates in cleavage of unit-length genomes and viral DNA packaging into preformed nucleocapsids (Nellissery et al., 2007). Our comparison of DEV UL5 sequences show that the different 15 amino acids at the aminoterminus of the 2085 UL6 protein do not functionally impair or functionally inactivate the protein.

3.4.2.6. Variations in other ORFs present in the unique-short genome segment

There are 3 genes that show frame-shift mutations in the US segment (US5, US7 and US10)(Fig 3.4). The absence of a cytosine at position 380 and a thymidine at position 381 in US5 of 2085 cause the initiation site being moved 423-bp downstream relative to that of the other viruses, thereby resulting in a truncation of 141 amino acids. There are also 2 non-synonymous substitutions (Asn to Ser and His to Tyr at position 67 and 147, respectively) in strain 2085 relative to the other strains (Supplementary table 2). This result indicates that even though the function of DEV US5 is unknown, a truncation of more than 26% protein sequence and two amino acid substitutions do not result in decreased virulence of 2085 or influence its ability to replicate in vitro or in vivo, but may be a characteristic of its geographic origin. The presence of near perfect Kozak sequences immediately upstream of the full-length and truncated US5 gene suggests that transcription and translation is likely from both predicted ORFs.

Regarding US7, Clone-03 has 100% amino acid similarity with CHv, and has only 1 non-synonymous substitution at position 1013, which causes a change of 1 amino acid when compared to 2085. However, the VAC strain exhibits a very high degree of nucleotide variation

from position 985 to 1039, which includes an alteration of 9 nucleotides resulting in 5 amino acids changes and one additional thymidine at position 1039 leading to an alteration of 25 amino acids that includes a deletion of 9 residues. There are also 4 non-synonymous substitutions in strain VAC (Ile to Asn, Ile to Asn, Arg to Leu and Thr to Ile in strain VAC at position 168, 295, 310 and 319, respectively)(Supplementary table 2). The products of US7 homologues of alphaherpesviruses encode glycoprotein I, a type I membrane protein associated with cell-to-cell spread that is non-essential for virus growth of most alphaherpesviruses other than MDV (Audonnet et al., 1990; Sullivan and Smith, 1988). Our comparative sequence analyses suggest that the mutations present in VAC may play an important role in the attenuation of the DEV VAC strain and present a potential target for molecular diagnostics to differentiate wild-type and vaccine strain VAC.

In the case of US10, which is predicted to encode a tegument phosphoprotein in other alphaherpesviruses, the VAC sequence has 100% identity with that of Clone-03, while the 2085 sequence is 100% identical with that of CHv. An absence of a thymidine at position 787 of 2085 and CHv shifts the reading frame and results in modification of the downstream 35 amino acids and an additional stretch of 11 amino acids. Because this polymorphism is identical in virulent and absent in vaccine strains, the modification at the carboxyterminus of strains VAC and Clone-03 may be a critical mutation in the process of attenuation.

The remaining 39 of the total 78 ORFs exhibit more than 99% identity between all 4 strains, where mutations result in amino acid substitutions (Supplementary table 2). It is worthwhile to note that DEV strain 2085 has "exclusive" strain-specific polymorphisms in 27 ORFs that result in a total of 57 amino acid substitutions relative to the other isolates, possibly an indicator of its geographic origin. Similarly, CHv has strain-specific polymorphisms in 8 ORFs resulting in 13 amino acid mutations. Surprisingly, Clone-03, a clonal derivative of strain VAC, has strain-specific polymorphisms in 12 ORFs including 25 amino acid alteration, a result that may indicate sequencing errors or point to a massive variability of DEV genome populations when propagated in CEC.

3.5. Concluding Remarks

In this study, the complete genome sequence of DEV strain 2085 was determined. The comparison with sequences of three other strains revealed that 30.8% (24/78) of the ORFs exhibit a 100% nucleotide sequence identity between strains. In addition, 50% (39/78) of the ORFs harbored nucleotide substitutions and shared more than 99% identity. 19.2% (15/78) of open reading frames exhibited sequence deletions and insertions resulting in frame-shift

mutations. The comparison also showed that 3 putative ORFs (UL2, UL12 and US10) with sequence deletions and 2 genes (UL47 and UL41) with non-synonymous substitutions are only present in two virulent strains, possibly pointing to their role in attenuation of the vaccine strains. However, considering the complexity of virulence of herpesviruses (Goodman et al., 2007; Spatz et al., 2007), further experiments are necessary to confirm their role in DEV virulence. In addition, we cannot exclude from the equation other open reading frames with polymorphisms that are only present in one of the virulent strains. Taken together, our results indicate that the virulent 2085 strain harbors markedly more specific polymorphisms (46.7% of highly variable ORFs and 69.2% of less variable ORFs) when compared to the other three strain, indicating lineage differences between European and Asian strains.

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Supplementary table 1. Comparison of the ORFs encoded by the DEV strain 2085 with VAC, Clone-03 and CHv[a].

Gene or ORF name/size(nt)	VAC Size(nt)	Identity	Reference	Clone-03 Size(nt)	Identity	Reference	CHv Size(nt)	Identity	Reference
LORF11/3171	828	22%	EU082088.2	2412	71%	EU294364.1			EU071034.1
LUL55/561	561	100%	EU082088.2	561	99%	EU294364.1	561	100%	FJ232030.1
LORF9/969	969	99%	EU082088.2	969	99%	EU294364.1	969	99%	EU071033.1
UL54/1134	1134	100%	EU082088.2	1134	99%	EU294364.1	1134	100%	EU071035.1
UL53/1005	1005	99%	EU082088.2	1032	99%[b]	EU294364.1	1032	99%[b]	EU071036.1
UL52/3375	3375	99%	EU082088.2	3375	99%	EU294364.1	3375	99%	DQ072725.1
UL51/759	759	99%	EU082088.2	759	99%	EF683581.1	759	99%	DQ486149.1
UL50/1344	1344	99%	EU082088.2	1344	99%	EF492886.1	1344	99%	EU195112.1
UL49.5/288	288	99%	EU082088.2	288	99%	EF492886.1	288	100%	EU195111.1
UL49/741	762	99%	EU082088.2	762	99%	EF492886.1	762	99%	EU195110.1
UL48/1425	1431	99%	EU082088.2	1425	99%	EF492886.1	1425	99%	EU195109.1
UL47/2364	2367	99%	EU082088.2	2367	99%	EF492886.1	2367	99%	EU195108.1
UL46/2220	2220	100%	EU082088.2	2220	100%	EF492886.1	2220	100%	EU195107.1
UL45/672	672	100%	EU082088.2	672	100%	EF492886.1	672	100%	
UL44.5/813	813	99%	EU082088.2	813	99%	EF683582.1			
UL44/1296	1296	100%	EU082088.2			FJ213607.1	1296	100%	EU076811.1
UL43/1503	1503	99%	EU082088.2	1503	99%	FJ213607.1	1503	99%	EU071037.1
UL42/1092	1092	99%	EU082088.2	1092	99%	FJ213607.1	1092	99%	EU071038.1
UL41/1494	1497	99%	EU082088.2	1497	99%	FJ213607.1	1497	99%	EU071040.1
UL40/1068	1068	99%	EU082088.2	1068	99%	FJ213607.1	1068	99%	EU071039.1
UL39/2433	2433	99%	EU082088.2	2433	99%	FJ213607.1	2433	99%	EU071042.1
UL38/1398	1068	100%	EU082088.2	1068	99%	FJ213607.1	1068	100%	EU071041.1
UL37/3201	3198	98%	EU082088.2	3201	99%	FJ213607.1	3201	99%	EU195088.1
UL36/10764	10764	99%	EU082088.2	10764	99%	EU422985.1	10764	99%	FJ222444.1
UL35/354	354	100%	EU082088.2	354	100%	EF203708.1	354	99%	EF643558.1
UL34/831	678	81%	EU082088.2	831	100%	EF203708.1	831	99%	EF643562.1
UL33/408	408	99%	EU082088.2	408	99%	EF203708.1	408	99%	EF643556.1
UL32/1962	1962	99%	EU082088.2			EF203708.1	1962	99%	EF643561.2
UL31/933	933	99%	EU082088.2	933	99%	EF203709.1	933	99%	EF643559.1
UL30/3621	3621	100%	EU082088.2	3621	100%	EF203709.1	3621	100%	EF643560.1
UL29/3585	3585	100%	EU082088.2	3585	100%	EF203709.1	3585	99%	EF643566.1
UL28/2412	2412	100%	EU082088.2	2412	100%	EF203709.1	2412	99%	EF643557.1
UL27/3003	3003	99%	EU082088.2	3003	99%	EF203709.1	3003	99%	EF608147.1
UL26.5/1074	1074	100%	EU082088.2	1074	100%	EF203709.1	1074	100%	EF643565.1

Continued

Gene or ORF name/size(nt)	VAC Size(nt)	Identity	Reference	Clone-03 Size(nt)	Identity	Reference	CHv Size(nt)	Identity	Reference
UL26/2124	2124	100%	EU082088.2	2124	100%	EF203709.1	2124	99%	EF643565.1
UL25/1797	1797	99%	EU082088.2	1797	99%	EF203709.1	1797	99%	EF643563.1
UL24/1230	1230	100%	EU082088.2	1230	99%	DQ227739.1	1230	100%	EF584450.1
UL23/1077	1077	100%	EU082088.2	1077	99%	AY963569.1			
UL22/2505	2505	100%	EU082088.2	2505	99%	DQ227740.1	2505	100%	EU195089.1
UL21/1686	1686	99%	EU082088.2	1686	99%	EF203707.1	1686	99%	EU195090.1
UL20/822	822	99%	EU082088.2	822	99%	EF203707.2	822	99%	EU195091.1
UL19/4143	4143	99%	EU082088.2	4143	99%	EF203707.2	4143	99%	EU195092.1
UL18/969	969	99%	EU082088.2	969	99%	EF203707.2	969	99%	EU195093.1
UL15b/1185	1185	100%	EU082088.2	1185	100%	EF203707.2			
UL17.5/309	309	100%	EU082088.2	309	99%	EF203707.2			
UL17/2055	2055	99%	EU082088.2	2055	99%	EF203707.2	2055	99%	EF643567.1
UL16/1089	1089	100%	EU082088.2	1089	100%	EF524094.1	1089	100%	EU195095.1
UL15A/1035	1035	99%	EU082088.2	1035	99%	EF524094.1	1035	99%	EU195094.1
UL14/465	465	100%	EU082088.2	465	100%	EF524094.1	465	99%	EU195097.1
UL13/1431	1539	93%	EU082088.2	1575	93%[b]	EF524094.1	1575	93%[b]	EU195098.1
UL12/1689	1452	86%	EU082088.2	1341	79%	EF524094.1	1689	100%	EU195099.1
UL11/264	264	100%	EU082088.2	264	100%	EF524094.1	264	100%	EU861976.1
UL10/1221	1221	99%	EU082088.2	1230	99%[b]	EF449515.1	1230	99%[b]	EU195100.1
UL9/2580	2580	99%	EU082088.2	2301	89%	EF449515.1	2580	99%	EU195101.1
UL8/2271	2271	99%	EU082088.2	2271	99%	EF449515.1	2271	99%	EU195102.1
UL7/966	966	100%	EU082088.2	966	100%	EF449516.1	966	100%	FJ222445.1
UL6/2388	2373	98%	EU082088.2	2373	98%	EF449516.1	2373	98%	EF055890.1
UL5/2568	2568	99%	EU082088.2	2568	99%	EF449516.1	2568	99%	EU195103.1
UL4/717	714	99%	EU082088.2	714	99%	EF449516.1	717	99%	EU195104.1
UL3.5/363	363	99%	EU082088.2	363	99%	EF449516.1	363	99%	EU195105.1
UL3/720	720	99%	EU082088.2	720	99%	EF449516.1	720	99%	EF608146.1
UL2/792	351	32%	EU082088.2	474	33%[b]	EF449516.1	1002	100%[c]	EU885419.1
UL1/11	711	99%	EU082088.2	711	99%	EF449516.1	711	100%	EU195106.1
LORF3/1410	1443	98%	EU082088.2	1443	98%	EU328306.1	1443	98%	EU861974.1
LORF2/1893	1893	99%	EU082088.2	1893	99%	EU328306.1	1893	99%	EU861973.1
IRS/4881	4881	99%	EU082088.2	4881	99%	HQ009801.1	4881	99%	EU195083.1
US1/990	990	100%	EU082088.2	990	100%	EF524095.1	990	100%	EU195087.1
US8/1473	1473	99%	EU082088.2	1473	99%	HQ009801.1	1473	99%	EU071044.1
US7/1116	1089	89%	EU082088.2	1116	99%	HQ009801.1	1116	99%	EU035298.1

Gene or ORF name/size(nt)	VAC Size(nt)	Identity	Reference	Clone-03 Size(nt)	Identity	Reference	CHv Size(nt)	Identity	Reference
US6/1269	1269	100%	EU082088.2	1269	100%	HQ009801.1	1269	100%	EU195085.1
US5/1197	1620	74%	EU082088.2	1620	74%	HQ009801.1	1620	74%	FJ222443.1
US4/1380	1380	99%	EU082088.2	1380	99%	HQ009801.1	1380	99%	EU071043.1

Continued

Gene or ORF name/size(nt)	VAC Size(nt)	Identity	Reference	Clone-03 Size(nt)	Identity	Reference	CHv Size(nt)	Identity	Reference
US3/1158	1158	100%	EU082088.2	1158	100%	HQ009801.1	1158	100%	EF584451.1
US2/720	720	99%	EU082088.2	720	99%	HQ009801.1	720	99%	EU195086.1
SORF3/888	888	99%	EU082088.2	888	99%	HQ009801.1	888	99%	EU861975.1
US10/930	897	81%	EU082088.2	936	81%[b]	HQ009801.1	969	100%[c]	EU195084.1

[a]The gene/ORF name and nucleotides of 2085 are listed. Designation, size, accession number of the gene/ORF in GenBank of indicated strains are given. Nuleotide similarity with strain 2085 is shown in percentage . ORFs that contain deletions, insertions or frame-shift mutations are in bold face.

[b]Size differences among the indicated strains due to different initiation sites, but no reasonable polymorphism observed for the initiation differences. Identity was determined after modification of initiation according to VAC strain

[c]Size differences among the indicated strains due to different initiation sites, but no reasonable polymorphism observed for the initiation differences.Identity was determined after modification of initiation according to 2085 strain

Empty spaces indicate that the sequence of these ORFs are not available.

Supplementary table 2. Single amino acid polymorphisms in ORFs[a]

Gene/ORF	2085	VAC	Clone-03	CHv	Gene/ORF	2085	VAC	Clone-03	CHv
LORF9 $_{305}$	Cys	Gly	Gly	Gly	UL55 $_{93}$	Val	Val	Ala	Val
UL54 $_{272}$	Lys	Lys	Arg	Lys	UL52 $_{33}$	Val	Val	Gly	Val
UL52 $_{325}$	Gly	Gly	Lys	Gly	UL52 $_{499}$	Asp	Asn	Asn	Asn
UL52 $_{515}$	Asp	Asp	Ala	Asp	UL52 $_{562}$	Leu	Leu	Val	Leu
UL52 $_{1123}$	Ser	Arg	Arg	Arg	UL48	/[b]	Met$_1$	/	/
UL48	/	Asp$_2$	/	/	UL48 $_{35}$	Gly	Gly	Ala	Gly
UL48 $_{62}$	Leu	Leu	Val	Leu	UL48 $_{203}$	Gly	Trp	Trp	Trp
UL48 $_{469}$	Ser	Leu	Leu	Leu	UL47 $_{36}$	Ala	Val	Val	Ala
UL47 $_{113}$	His	Arg	Arg	Arg	UL47 $_{148}$	Δ[c]	Asp	Asp	Asp
UL47 $_{599}$	Ala	Thr	Thr	Thr	UL44.5 $_{241}$	Ile	Val	Val	
UL44.5 $_{250}$	Asp	Asn	Asn		UL43 $_{389}$	Gly	Val	Val	Val
UL43 $_{464}$	Met	Lys	Lys	Lys	UL43 $_{499}$	Gly	Ser	Ser	Ser
UL42 $_{146}$	Asp	Asp	Gly	Asp	UL42 $_{169}$	Thr	Thr	Ala	Thr
UL42 $_{209}$	Gly	Glu	Glu	Glu	UL41 $_{44}$	Glu	Gly	Gly	Glu
UL41 $_{125}$	Δ	Asp	Asp	Asp	UL41 $_{302}$	Cys	Tyr	Tyr	Tyr
UL40 $_{10}$	Gly	Ser	Ser	Ser	UL39 $_{204}$	Ile	Val	Val	Val
UL39 $_{452}$	Tyr	Asp	Asp	Asp	UL39 $_{538}$	Ile	Ile	Val	Ile
UL37 $_5$	Gly	Asp	Asp	Asp	UL37 $_{170}$	Lys	Ile	Lys	Lys
UL37 $_{385}$	Asn	Thr	Thr	Thr	UL36 $_{493}$	Leu	Leu	Pro	Leu
UL36 $_{528}$	Arg	Lys	Lys	Lys	UL36 $_{643}$	Thr	Thr	Ile	Thr
UL36 $_{713}$	Asn	Asn	Asp	Asn	UL36 $_{769}$	Asn	Asn	Asp	Asn
UL36 $_{936}$	Gly	Asp	Asp	Asp	UL36 $_{1095}$	Thr	Ser	Ser	Ser
UL36 $_{1466}$	Gly	Gly	Arg	Gly	UL36 $_{1834}$	Ala	Val	Val	Val
UL36 $_{1982}$	His	His	His	Gln	UL36 $_{2125}$	Asn	Asn	Ser	Asn
UL36 $_{2208}$	Ala	Thr	Thr	Thr	UL36 $_{2481}$	Asp	Thr	Thr	Thr
UL36 $_{2919}$	His	Thr	Thr	Thr	UL36 $_{2960}$	Asn	Asn	Ser	Asn
UL36 $_{3132}$	Val	Ala	Ala	Ala	UL36 $_{3202}$	His	Pro	Pro	Pro
UL36 $_{3203}$	Arg	Trp	Trp	Trp	UL36 $_{3210}$	Gly	Gly	Arg	Gly
UL36 $_{3308}$	Arg	Cys	Cys	Cys	UL36 $_{3474}$	Ser	Phe	Phe	Phe
UL36 $_{3498}$	Gly	Ser	Ser	Ser	UL34 $_{124}$	Leu	Leu	Leu	Ser
UL31 $_{121}$	Pro	Pro	Ser	Ser	UL31 $_{161}$	Gln	Gln	Gln	Glu
UL29 $_{314}$	His	His	His	Gln	UL29 $_{1099}$	Glu	Glu	Glu	Asp
UL28 $_{559}$	Ala	Ala	Ala	Ser	UL27 $_{891}$	Met	Met	Met	Val
UL26 $_{30}$	Arg	Arg	Arg	Thr	UL26 $_{103}$	Val	Val	Val	Glu
UL26 $_{149}$	Glu	Glu	Glu	Gln	UL25 $_{18}$	Thr	Ala	Ala	Ala
UL25 $_{37}$	Trp	Trp	Trp	Cys	UL25 $_{39}$	Lys	Lys	Arg	Arg
UL25 $_{171}$	Ser	Ser	Ser	Ile	UL25 $_{267}$	Phe	Phe	Phe	Ser
UL25 $_{598}$	Met	Met	Met	Ile	UL23 $_{267}$	Val	Val	Ala	
UL22 $_{508}$	Leu	Leu	Pro	Leu	UL21 $_{253}$	Gly	Glu	Glu	Glu
UL21 $_{268}$	Leu	Leu	Pro	Leu	UL21 $_{436}$	Ala	Ala	Val	Ala
UL21 $_{447}$	Lys	Lys	Gly	Lys	UL20 $_{47}$	Ala	Thr	Thr	Thr
UL19 $_{465}$	His	His	Arg	His	UL19 $_{728}$	Ile	Ile	Val	Ile
UL19 $_{929}$	Arg	Ser	Ser	Ser	UL19 $_{1147}$	Gly	Asp	Asp	Asp
UL19 $_{1180}$	Tyr	Ser	Ser	Ser	UL18 $_{86}$	Ala	Val	Val	Val
UL17.5 $_3$	Cys	Cys	Arg		UL17 $_{167}$	Arg	Trp	Trp	Trp
UL17 $_{343}$	Asp	Gly	Gly	Gly	UL17 $_{657}$	Asp	Gly	Gly	Gly
UL15A $_{136}$	Arg	His	His	His	UL10 $_{23}$	Ser	Trp	Trp	Trp
UL10 $_{126}$	Arg	His	His	His	UL10 $_{170}$	Val	Ile	Val	Val
UL6	Pro$_{379}$	Ser$_{274}$	Ser$_{374}$	Ser$_{374}$	UL5 $_{278}$	Ser	Phe	Ser	Ser
UL5 $_{346}$	Met	Thr	Thr	Thr	UL5 $_{599}$	Lys	Lys	Ile	Lys
UL5 $_{717}$	Ile	Thr	Thr	Thr	UL4 $_{87}$	Lys	Δ	Δ	Δ

Continued

Gene/ORF	2085	VAC	Clone-03	CHv	Gene/ORF	2085	VAC	Clone-03	CHv
UL4 176	Δ	Δ	Δ	Glu	UL3.5 51	Gly	Trp	Trp	Trp
UL3 38	Gly	Val	Val	Val	UL2	Gly193	Val24	Gly87	Gly263
UL1 231	Gly	Val	Gly	Gly	LORF3 25	Thr	Ala	Ala	Ala
LORF3	Glu317	Asp328	Asp328	Asp328	LORF2 472	Thr	Ser	Ser	Ser
LORF2 509	Gly	Ls	Lys	Lys	IRS 153	Ile	Val	Val	Val
IRS 153	Ala	Val	Val	Val	IRS 1073	Ser	Thr	Thr	Thr
IRS 1159	Lys	Glu	Glu	Glu	IRS 1204	Arg	Gly	Gly	Gly
US8 48	Thr	Ile	Ile	Ile	US8 138	Ile	Val	Val	Val
US8 451	Ser	Pro	Pro	Pro	US7 168	Ile	Asn	Ile	Ile
US7 295	Ile	Asn	Ile	Ile	US7 310	Arg	Leu	Arg	Arg
US7 319	Thr	Ile	Thr	Thr	US7 329	Ser	Pro	Ser	Ser
US7 332	Asp	His	Asp	Asp	US7 337	Gln	Leu	Gln	Gln
US7 338	Gly	Tyr	Asp	Asp	US7 343	Lys	Gln	Lys	Lys
US5	/	Arg108	Met108	Arg108	US5	Ser67	Asn208	Asn208	Asn208
US5	Tyr 147	His288	His288	His288	US4 14	Arg	Cys	Cys	Cys
SORF3 269	Val	Ile	Ile	Ile					

[a]Changes in DEV 2085, VAC, Clone-03 and CHv are shown. Position of non-synonymous amino acid substitutions are indicated for all four strains.

[b]indicates that no predicted amino acid is encoded at that site. Empty spaces indicate that sequences are not available.

[c]absence of a codon and amino acid.

Chapter 4. Discussion

4.1. Construction of a DEV BAC

Viral bacterial artificial chromosome clones (BAC) are constructed by insertion of a BAC vector, a low copy *Escherichia coli* F-factor plasmid that exists in individual host cells in a supercoiled circular form. The F-factor allows stable maintenance of individual DNA molecules as well as easy manipulation of the cloned DNA in *Escherichia coli* cells, and the insertion size of nucleotide fragment can be as big as 700-kbp (Stone et al., 1996). The F-factor based plasmids have been further modified by deletion of sequences that are non-essential for its function and by adding of some selection or marker genes such as the chloramphenicol resistance gene and a GFP expression cassette, resulting in the so-called mini-F factor. The BAC system was developed initially for the Human Genome Project and subsequently used in many other genome projects. The technique has expanded the possibilities for studying functional genetics in vitro and in vivo more efficiently (Narayanan and Chen, 2011; Shizuya and Kouros-Mehr, 2001).

The use of BAC technology in herpesvirus genetics has made their genomes accessible to the arsenals of bacterial genetical engineering. The fact that herpesvirus genomes circularize during replication makes feasible transformation of virus DNA into *E. coli cells* via electroporation. This technique also allows the efficient reconstitution of viral progeny by transfection of the BAC plasmid DNA into eukaryotic cells. After the murine cytomegalovirus (MCMV) BAC was constructed in 1997, more and more BACs of herpesviruses were generated, opening up new avenues for delineating the functions of viral genes, understanding the pathogenesis of their associated diseases and also for vector and vaccine development or gene therapy (Adler et al., 2003; Baigent et al., 2006; Brune et al., 2000 ; Horsburgh et al., 1999; Messerle et al., 1997; Robinson et al., 2008; Warden et al., 2011; Zhou and Gao, 2011).

The method commonly used to generate herpesvirus BACs is by insertion of the Mini-F factor through traditional homologous recombination to replace a non-essential gene in the virus genome or introduction of the vector without any replacement. For example, oncogenic and an avirulent strains of MDV were cloned as BACs by replacement of US2 with the Mini-F vector. The generation of BACs of bovine herpesviruses type 1 (BHV-1) and type 5 (BHV-5) and guinea pig cytomegalovirus (GPCMV) was also performed by replacement of fragments identified with restriction enzymes digestion (Cui et al., 2009; Cui et al., 2008; Gabev et al., 2009; Smith et al., 2011). Some other herpesviruses, which were constructed as BACs without gene replacement

but only with insertion of the Mini-F vector, include murine gammaherpesvirus-68 (MHV-68) where the vector was placed at the left end of M1 ORF, feline herpesvirus-1 (FHV-1) at the left end of UL, bovine herpesvirus 4 (BHV-4) at the intergenic region between ORF2 and ORF3, and equine herpesvirus 4 (EHV-4) in the intergenic region between gene 58 and 59 (Azab et al., 2009; Donofrio et al., 2009 ; Tai et al., 2010; Wu et al., 2011). Even though most herpesvirus were shown to be tolerant to large foreign DNA fragments, some herpesviruses, for instance human cytomegalovirus (HCMV) and MCMV, have a limited packaging capacity and insertion of the appropriate 10-kbp Mini-F vector may severely impair the growth of these viruses. In these cases, replacement of a long fragment of the genome is not optional but necessary. Other than these two common methods through traditional recombination, BACs were also generated from overlapping cosmids as was done successfully for VZV (Tischer et al., 2007), simian varicella virus (SVV) (Gray et al., 2011), and another BAC was generated by direct cloning of unit length genomes for human herpes virus 6A (HHV-6A) too, a method optimal for viruses that replicate inefficiently (Borenstein and Frenkel, 2009).

In the construction of BAC of DEV in our study, we employed the traditional recombination method by replacement of the UL44 (gC) gene. This strategy was based on the facts that UL44 homologs of alphaherpesviruses are non-essential for virus replication and DEV can grow very well on CECs. The successful generation of the DEV BAC proved the capacity of packaging extra DNA sequences, indicating the potential of DEV to serve as vector to harbor at least 8-kpb of foreign DNA sequence. When compared to direct cloning or generation from overlapping cosmids like that of VZV, SVV and HHV-6A (Borenstein and Frenkel, 2009 ; Gray et al., 2011), the traditional homologous recombination method employed in this research showed high efficiency. Besides, another advantage is that, along with the construction of the BAC, a gC deletion mutant was generated at the same time, which provided a way to study the function of gC without further mutagenesis. However, the result that deletion of gC affected the titers of supernatant and intracellular significantly demonstrate that anther option of insertion of Mini-F without any replacement is needed when the growth ability of the BAC derived virus is not to be affected. In this case, the intergenic region between LORF2 and LORF3 seems to be an optimal place because this interval is as long as 2,202 base pairs without any overlapping open reading frame (see chapter 3).

4.2. Applications of the engineered DEV BAC

Mutagenesis using herpesvirus BAC technology has shown to be an invaluable tool for pathogenesis research of herpesviruses. One popular method for manipulating BACs of

herpesvirus is site-directed mutagenesis, which utilizes homologous recombination to generate specific mutations in one or multiple viral genes at one time. For example, functions of most of the genes of HSV-1 and 70 unique ORFs of VZV have been studied using this approach. Other herpesviruses and their genes were investigated recently that way, too, which include ORF 10 of SVV, ORF11 of murine MHV-68, K8 and ORF65 of Kaposi's sarcoma-associated herpesvirus (KSHV), rhBARF1 of rhesus macaque lymphocryptovirus (rhLCV) and the gE of gallid herpesvirus 3 (GaHV-3)(Gray et al., 2011; Ohashi et al., 2011; Petherbridge et al., 2009; Sathish and Yuan, 2010 ; Smith et al., 2011; Wang et al., 2011b; Zhang et al., 2010). In this study, we have obtained a DEV gC deletion mutant through this site-directed way and it is obviously very useful for further research of other genes or ORFs of DEV based on the BAC generated here.

Transposon mutagenesis is another applicable method for BAC manipulation (Brune et al., 1999; Hobom et al., 2000; McGregor et al., 2004; Robinson et al., 2008). This type of mutagenesis is random and can cause a large diversity of BAC mutants which would provide a global approach to deletions of viral genes and help indentify essential and non-essential genes or host tropism factors on a whole genome scale (Warden et al., 2011). But the disadvantages of this method include: (1) location of the transposon insertion site needs to be identified, (2) the "hit probablility" of remaining ORFs will decrease along with the number of ORFs already hit, (3) insertion in the middle of an ORF may cause expression of a partial yet functional protein making the results unclear (Warden et al., 2011). In any case, transposon mutagenesis can produce a large number of mutants in a short time. Perhaps if one combined this method with site-directed mutagenesis, global gene analysis of DEV could be more convenient.

Another herpesvirus BAC application includes the generation of gene-deleted herpesvirus vaccines and vectored vaccine. One of the most successful applications of a gene-deleted herpesvirus vaccine in animals was seen during the eradication campaign for pseudorabies in U.S. and European domestic swine population. The eradication was accomplished through the use of glycoprotein E (gE) deleted modified live virus vaccines and an accompanying gE differential enzyme-linked immunosorbent assay (ELISA) (Ferrari et al., 2000 ; Ma et al., 2008; Meeusen et al., 2007). Another gE gene-deleted marker vaccine for BHV-1 was also licensed and is in widespread use in the EU (Meeusen et al., 2007). An MCMV modified live virus vaccine derived from a genome cloned as a BAC seemed to open the door to clinical trials of safer and more immunogenic HCMV vaccines (Schleiss, 2010). MEQ gene deletion mutants of MDV also based on virus genomes cloned as BACs showed a promising potential for the development of new generation vaccines against MDV strains with increasing virulence (Silva et al., 2010). For

the control of duck plague, a gene deletion vaccine would be also an optimal option for making possible differentiation of antibodies induced by vaccination or by infection with wild-type virus. And the successful construction of a DEV BAC reported here will provide a convenient way to conduct future research of gene deletion marker vaccines against DEV.

Vectored vaccines with herpesvirus as the carrier backbone have shown another promising usage of herpesvirus improved by the generation of BACs. HVT-vectored VP2 chimera vaccines against MD and infectious bursal disease (IBD) have already been licensed (Darteil et al., 1995; Meeusen et al., 2007; Tsukamoto et al., 2002). ILTV recombinants expressing H5 hemagglutinin and N1 neuraminidase can provide protection against H5N1 highly pathogenic avian influenza virus infection (Pavlova et al., 2009; Veits et al., 2003). EHV-1 BACs have been used for generation of vectored vaccines to induce immune response against equine influenza virus (EIV), canine influenza virus (CIV), bovine viral diarrhea virus (BVDV) and others (Rosas et al., 2007; Rosas et al., 2008; Van de Walle et al., 2010). In this study, a proof-of-principle method has been performed to analyze the potential of DEV to serve as a vector to express foreign proteins. Even though further animal challenge studies still need to be conducted for evaluation as to the immunogenicity of the H5 recombinants in vivo, IIF and western blotting test have proved the rebust expression of H5 in vitro, a result indicating a promising potential of DEV vectored vaccines. When this result is correlated with the tolerence of up to 8-kbp extra DNA in the DEV genome, construction of multivalent recombinant vaccines based on DEV BAC seems possible.

4.3. Glycoproteins of DEV

Glycoproteins of herpesviruses, which localize in the virion envelope, play important roles in virus entry into and egress from host cells. There are 12 glycoproteins predicted to be encoded by DEV (Li et al., 2009). All these proteins have homologs in HSV-1 or MDV, except for the gD and gJ genes in MD (Pellett and Roizman, 2007; Tulman et al., 2000). Studies have shown the HSV-1 of UL49.5 (glycoprotein N, gN), UL27 (glytoprotein B, gB), UL22 (glycoprotein H, gH), UL1 (glycoprotein L, gL) and US6 genes (glycoprotein D, gD) are essetial for virus replication, while the other seven glycoproteins including UL53 (glycoprotein K, gK), UL44 (glycoprotein C, gC), UL10 (glycoprotein M, gM), US4 (glycoprotein G, gG), US5 (glycoprotein J, gJ), US7 (glycoprotein I, gI) and US8 (glycoprotein E, gE) are non-essential (Pellett and Roizman, 2007).

Some of the glycoprotein homologs of MDV, BHV-1, PRV and other *Alphaherpesvirinae* have been studied (Chattoo et al., 2006; Mettenleiter, 2000; Robinson et al., 2008; Schumacher et al., 2000; Zhang et al., 2010)(Table 4.1). Amino acid sequence analysis of MDV gK shows some

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common glycoprotein features, but up to date no report is available on whether it is essential or non-essential for MDV. An essential function of gK in virus egress but not entry was demonstrated for PRV (Klupp et al., 1998; Ren et al., 1994). gN encoded by the varicelloviruses also were shown to be capable of inhibiting TAP and the gN homologous gene is essential for MDV growth in cultured cells (Tischer et al., 2002 ; Verweij et al., 2011). Studies showed that gC is important for horizontal spread of MDV between chickens and to cell-to-cell spread capabilities of the virus (Jarosinski and Osterrieder, 2010; Tischer et al., 2005). Glycoproteins C of MDV expressed by baculovirus recombinants can induce antibody against MDV, but can not protect against challenge with virulent virus (Jang et al., 1996). Other studies show that gC of BHV-1, HSV-1, PRV, ILTV, and VZV are all non-essential (Pavlova et al., 2010). MDV gB also is essential for virus growth and its expression by a recombinant fowlpox or baculo viruses resulted in protection against lethal challenge, indicating a strong immunity-inducing ability of MDV-1 gB (Jang et al., 1996; Kato et al., 1999; Liu et al., 1999 ; Omar et al., 1998 ; Yoshida et al., 1994). One study showed that MDV gL may be required for proper processing and transport of gH to the cell surface (Wu et al., 2001; Yoshida et al., 1994). Domain peptides derived from MDV gH were able to significantly reduce lesion formation on chorioallantoic membranes (CAMs) of infected chicken embryos, indicating its immunogenicity. However, no gH or gL deletion mutants were constructed to check whether they are indispensable for MDV replication (Wang et al., 2011a). Naturally occurring deletion or substitute mutations within the gL gene of MDV field isolates or very virulent (vv)+MDVs were apparently selected through Marek's disease vaccination, indicating that gL seems to possibly be altered in the evolution of MDV towards greater virulence (Santin et al., 2006; Tavlarides-Hontz et al., 2009; Wu et al., 1999). Different from HSV-1 or BHV-1, glycoprotein M of MDV is indispensable for cell-to-cell spread (Cai et al., 1999; Osterrieder, 1999; Tischer et al., 2002). The gJ gene is absent in the genomes of VZV, BHV-1, PRV and MDV. A study showed that deletion of gJ of ILTV has only minor effects on direct cell-to-cell spread but titers were significantly reduced (Fuchs et al., 2005). MDV gD is non-essential for virus growth and lacks many important functions that are characteristic for other alphaherpesviral gD homologues. It is expressed in a very limited set of cells and expression may be differentialy regulated in various cell types, which results in the fact that MDV gD is not required for oncogenicity or horizontal transmission. MDV seems to be an intermediate type of alphaherpesvirus when compared to the highly gD-dependent herpes simplex virus and the other extreme of the varicella-zoster virus which lacks the gD gene (Anderson et al., 1998; Niikura et al., 1999; Ono et al., 1996 ; Parcells et al., 1994; Tan et al., 2001; Zelník et al., 1999). gE and gI of MDV are essential for cell-to-cell spread, a characteristic

different from the non-essential property of the homolog of HSV-1 (Ross and Binns, 1991; Schumacher et al., 2001; Schumacher et al., 2002). The licensed gE deletion live virus vaccines of PRV and BHV-1 show that gE of both these two virus are non-essential, just as that of HSV-1, and that deletion of gE does not affect the properties of the mutants as vaccine candidates (Ferrari et al., 2000 ; Ma et al., 2008; Meeusen et al., 2007).

The fact that relatively high titers of serum antibodies against viral glycoproteins are induced in herpesvirus-infected animals together with the non-essential character of some glycoproteins make the use of glycoprotein gene-deletion vaccines an optimal choice. This kind of gene-deletion marker vaccines can play an important role in the eradication of herpesvirus diseases because it can provide a way to differentiate vaccinated animals from field-infected animals.

Table 4.1. Essentiality of glycoprotein homologues of members of the *Alphaherpesvirinae*.

Gene/protein	DEV	MDV-1	ILTV	PRV	BHV-1	VZV	HSV-1
UL53/gK	NA	NA	NA	E	E	E	N
UL49.5/gN	NA	E	N	N	N	E	E
UL44/gC	N	N	N	N	N	N	N
UL27/gB	NA	E	NA	E	E	E	E
UL22/gH	NA	NA	NA	E	E	E	E
UL10/gM	NA	E	NA	N	N	E	N
UL1/gL	NA	NA	NA	E	E	E	E
US4/gG	NA	SORF4	NA	N	N	AB	N
US5/gJ	NA	AB	N	AB	AB	AB	N
US6/gD	NA	N	NA	E	E	AB	E
US7/gI	NA	E	NA	N	N	DE	N
US8/gE	NA	E	NA	N	N	E	N

NA, no available information about essential or non-essential character ; N, non-essential; E, essential; AB, absence of the gene; DE, defect.

Glycoprotein gene deletion live vaccines of DEV could potentially promote the control of duck plague by capitalizing on these advantages as well. Our study showed that gC is a non-essential gene for DEV. However the deletion of gC significantly affects virus titers in the supernatant and infected cells, which is a clear disadvantage for vaccine production where efficient growth of the vaccine viruses is imperative. An overview of the essential or non-essential nature of the glycoprotein homologues in some members of *Alphaherpesvirinae* (Table 4.1) indicates that gG, gJ, gD and gE are further candidates to be deleted for generation of marker vaccine.

4.4. A DEV-vectored H5 Vaccine Candidate

Vaccination is a very important component in the control against HPAIV H5N1. Several HPAIV H5N1 vaccines have been developed and some are in use in the field already (Loulergue and Launay, 2009; Swayne, 2006). For example, the Chinese government has pursued a vaccination strategy in poultry, mainly with inactivated H5N1 vaccines. This strategy has

successfully reduced the incidence of H5N1 infection in commercial poultry in China (Chen, 2009; Chen and Bu, 2009). Numerous studies showed that recombinant canarypox virus, raccoonpox virus and HVT expressing hemagglutinin (HA) of influenza virus A (H5N1) can induce protective immunity, indicating vectored vaccines are promising alternatives in the control of HPAIV (Hwa et al., 2010; Rauw et al., 2011; Stittelaar et al., 2010). An H5N1 cold-adapted attenuated vaccine virus has been developed as well and was shown to fully protect mice and ferrets four weeks after application of two doses of the vaccine (Suguitan et al., 2006). Recombinant influenza virus vaccine candidates generated by fusing the HA1 fragment of A/Anhui/1/2005(H5N1) to either Fc of human IgG (HA1-Fc) or to foldon plus Fc (HA1-Fdc) were constructed. These vaccines were shown to provide cross-protection against infections with divergent strains of highly pathogenic H5N1 virus (Du et al., 2011).

As ducks play a crucial role in the epidemiology of HPAIV H5N1, vaccination of ducks is also an important measure to take. The immunogenicity and protective efficacy of a CD40-targeting avian influenza virus HA subunit DNA vaccine were examined in ducks and the results suggested that CD40-targeting holds promise for influenza A vaccine development (Yao et al., 2010). Another study has shown that protection against HPAIV challenge can be induced for domestic ducks and geese inoculated with inactivated vaccines. But a single dose of this vaccine failed to provide adequate protection for ducks or geese and was thus less effective as it was in chickens (Eggert and Swayne, 2010).

The promising potential of DEV to serve as a vector to express hemagglutinin of HPAIV H5N1 shown in this study provides evidence that construction of a DEV vectored H5 vaccine is possible. Given that DEV vaccine administration is a routine inoculation in duck flocks, this kind of chimeric vaccines would make possible protection against both duck plague and HPAI with only one vaccination. If we were able to combine a gene-deleted marker attenuated live DEV vaccine with the vectored H5, the resulting vaccine would provide a near perfect vaccine against both diseases as vaccinated animals could be differentiated from field-infected animals in both cases by suitable serological assays. Such assays are already in use for HPAIV but would have to be developed for DEV.

4.5. Comparison of DEV Genomes

Comparison of the genomes of herpesvirus can provide a 'global' perspective for understanding the pathogenicity and geographic lineage of viruses. Several studies focused on comparison of complete genomes of different MDV strains or with HVT have yielded important imformation of the oncogencity of MDV. The comparison between the whole genome of HVT, MDV and GaHV-

3 indicated that the pp38 and *meq* genes, which are missing in HVT and GaHV-3, may play important roles in the pathogenesis and the evolution of more virulent MDV strains (Kingham et al., 2001). The complete sequence of the GaHV-3 SB-1 strain, which is a widely used vaccine strains against used in the protection against MD, was compared with another GaHV-3 strain, HPRS24. The results of the genomic comparison revealed that the SB-1 genome has greater sequence identities and similarities to homologous ORFs in the MDV genome than with those of HPRS24. Additionally, 19 ORFs differ in length and between the two GaHV-3 strains, indicating general polymorphisms that may be related to immunogenicity of certain GaHV-3 strains (Spatz and Schat, 2011). Comparison of the genome sequences of a BAC-derived RB-1B virus with a "non-spreading phenotype" with those of Md5, Md11, CVI988 and parental RB-1B provided useful data with respect to the pathogenicity and oncogenicity of MDV (Spatz et al., 2007). The complete genome sequences of two BAC clones of MDV and of the parent virus, the very virulent UK isolate C12/130, were also determined, and the comparison suggested that the C12/130 virus population likely exists as a collection of mixed genotypes (Spatz et al., 2011). Other studies showed that even though *meq* deletion can attenuate MDV efficiently, presence of the oncoprotein alone is not a priori a virulence factor as highlighted by the fact that the CVI988/Rispens vaccine virus still contains the gene and hence is not easily distinguishable, antigenically or genetically, from virulent MDV. Taken together, the results with respect to MDV have shown that attenuation of MDV is multifactorial process and not easy to address (Ajithdoss et al., 2009; Baigent et al., 2011; Kim et al., 2010 ; Silva et al., 2010).

The comparison of the genome sequences of two virulent DEV strains (2085 and CHv) and two vaccine DEV strains (VAC and Clone-03) in our study showed that there were not complete genes missing in the vaccine strains, although quite remarkable truncations and frame-shift mutations were identified. When we take into account the fact that both DEV vaccine strains were attenuated by serial passages on CECs, we may find that serial passages not only resulted in attenuation but also increased replication ability in CECs significantly. Robust replication, however, is an important property for a vaccine virus candidate, but unfortunately lost in most gene deletion mutants. It can be supposed that the loss of full function of intact open reading frame(s) would influence growth property significantly, but partial loss perhaps can avoid sublethal damage, and, meanwhile the partial deleteion mutant is attenuated along with the generation of a potential marker. The study on DEV genomes and growth kinetics of DEV gC deletion mutants supplied evidence to support this hypothesis, providing us with useful data for the rational design of DEV nucleotide deletion marker vaccines, DEV vectored vaccines and DEV chimeric vaccines.

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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 Anspruch genommen habe.

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