

博士論文

**Establishment of Marek's disease virus vector vaccine and its
application**

(マレック病ウイルスベクターワクチンの開発とその応用)

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GENERAL INTRODUCTION

I. Recombinant vector vaccines

The concept of vaccination was first reported in the late 18th century by Edward Jenner (23), who used a live-attenuated vaccine against the smallpox virus. Since then, live-attenuated vaccines have been the mainstay in the field of vaccination. Live attenuated vaccines are prepared from bacteria or viruses that are infectious, but have decreased or no pathogenicity. These vaccines are prepared by serially passaging the pathogens in cell cultures or animals until their virulence reduces. The main advantage of these vaccines is the strong, long-lasting immunity that they elicit; they can induce both humoral immunity and cellular immunity and are also cost effective. In addition, reversions and unintended malfunctions in vaccinated animals are relatively rare. In contrast, inactivated vaccines do not have virulence. They have zero infectivity and are therefore safer than live-attenuated vaccines. Furthermore, inactivated vaccines can induce faster onset of immunity; however, they elicit only humoral immunity and therefore have lower immunogenicity than live-attenuated vaccines. Live-attenuated vaccines and inactivated vaccines are the most commonly used vaccines and are generally considered as the classical vaccines. Apart from these, new kinds of vaccines, such as recombinant vector

vaccines, subunit vaccines, and conjugate vaccines, have been developed recently (18)(38).

Recombinant vector vaccines are live-attenuated vaccines that carry inserted foreign genes in their genomes. They can elicit immunity against the inserted antigens in addition to the pathogens themselves and are thus beneficial in providing protection against multiple agents of a disease. They can provide strong, long-lasting immunity because they derive from live-attenuated vaccines. In addition, they are useful to differentiate between infected and vaccinated animals. Recombinant vaccines are prepared by genetic engineering. Genetic engineering on viruses was performed for the first time in poxviruses in the 1980s (53). It has since been used for modifying adenoviruses, herpesviruses, and RNA viruses (52). Today, the market for recombinant vaccines is growing, and it constitutes a big part of the animal vaccine industry.

II. Recombinant vector vaccines for poultry

Chicken is one of the most widely consumed meat products in the world owing to the fact that there are no religious constraints prohibiting its consumption. The superior feed conversion and the low prices have also helped expand its market. However, the low prices have made it challenging to cover the costs of disease prevention, i.e., the cost of

injections, vaccines, medicines, and so on. Hence, the availability of effective and affordable vaccines is important for the poultry industry. In this regard, recombinant vaccines can be useful as they can provide immunity against multiple diseases with one strain of virus. Moreover, a single dose is sufficient to elicit a strong immune reaction in some recombinant vaccines.

The poultry industry has to deal with many pathogens such as avian influenza virus (AIV), Marek's disease virus (MDV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), *Salmonella*, and *Escherichia coli*. A number of vaccines, including recombinant vector vaccines, have been developed against these pathogens. For recombinant vector vaccines for poultry, herpesvirus of turkeys (HVT), adenovirus, NDV, and fowlpox virus are the commonly used vectors, and of these, HVT is the most commonly used.

III. Marek's disease virus

MDV, also called *Gallid alphaherpesvirus 2*, belongs to the family *Herpesviridae*, the subfamily *Alphaherpesvirinae*, and the genus *Mardivirus* (Fig. 1). Its genome comprises a double-stranded linear DNA of 160 to 180 kb, containing more than 70 genes in four regions: unique long (UL), unique short (US), terminal repeat (TR), and internal repeat

(IR) (Figs. 2 and 3). Three serotypes of MDV exist, and only serotype 1 (MDV1) is oncogenic and virulent. Serotype 2 (MDV2) is naturally apathogenic, while serotype 3 (MDV3) is the HVT strain that is used for vaccination.

MDV persistently infects T-cells of chickens and causes T-cell lymphoma, called Marek's disease (MD). The virus is cell-associated, and enveloped infectious virions are observed only in feather follicles, where the mature viruses bud. MD is a highly contagious oncogenic disease. Infected chickens show depression, paralysis in their legs and wings, and develop lymphoid tumors in the nerves. Mortality rate of MD varies from 1% to 50% depending on the condition of the flocks and the infectious strain. MD was first described in 1907 (34), and it caused huge economic damages worldwide until the 1970s, when the first vaccine with live apathogenic MDV was developed. Currently, all serotypes of MDV—attenuated variants of MDV1, MDV2, and HVT—are being used as vaccines. While these vaccines provide protection against MDV, they cannot prevent infection. Therefore, MD occurrence remains a ubiquitous problem worldwide.

IV. Herpesvirus of turkeys

HVT, also called *Meleagrid alphaherpesvirus 1*, belongs to MDV serotype 3. It was first isolated from turkeys and is not pathogenic to chickens. Unlike MDV1 and MDV2

vaccines, it can be prepared in both cell-free and cell-associated forms, and it develops bigger plaques (Fig. 4), which improves the economic feasibility of vaccine production as well. It has been widely used, alone or in combination with MDV1 or MDV2, as a vaccine against MDV since the 1970s. In addition, it has been used as a vector for recombinant vaccines because of its capacity to accommodate foreign genes, ability for persistent infection, and safety. HVT vector vaccines are commercially available from several companies. HVTs expressing antigen genes of IBDV, NDV, ILTV, or AIV are sold in many countries and have a large market; however, there are no vaccination programs that combine different HVT vector vaccines. Moreover, HVT alone cannot protect chickens from “very virulent MDV” and “very virulent plus MDV” strains; these strains of MDV have been observed since the 1980s, and MDV1 or MDV2 vaccines have been used in combination with HVT to enhance the protective efficacy against these strains. It should be noted that the MDV1 Rispens strain has been used with HVT and has been reported to be safe and effective if used in combination with HVT vector vaccine (29). However, there is no combination strategy for HVT with MDV1 or MDV2 vector vaccines. In Chapter 2, the author discusses a strategy for combined usage of HVT and MDV1 vector vaccines and proposes a new vaccination strategy against MDV and two other pathogens.

V. MDV1 Rispens strain

The Rispens strain, also called CVI988, belongs to MDV serotype 1. It has been used as a vaccine against MDV since the early 1970s and is the most effective vaccine against very virulent MDV strains as well. It was isolated from a flock of chickens in 1972 (46) and was passaged in duck embryo fibroblasts for conversion into an avirulent form. Since then, it has been used alone or in combination with HVT for prevention of MDV. Like HVT, it infects the T-cells of chickens and induces long-lasting immunity. However, it is propagated only in the cell-associated form and shows slower growth than HVT, making it less cost-effective.

The Rispens strain has also been suggested for use as a vector. Studies on the Rispens vector vaccine have been performed since the 1990s (48). In these studies, antigen genes of IBV (67), IBDV (30)(69), or NDV (55) were inserted into the Rispens genome and effective protection was reported. However, there are no commercially available Rispens vector vaccines in the market today. In Chapter 1, the author discusses the characteristics and properties of the Rispens vector vaccine aiming at its commercial release.

VI. Excision of bacterial artificial chromosome-derived sequences from recombinant virus

To construct recombinant herpesviruses, the method of homologous recombination in eukaryotic cells was frequently used. However, this approach involves laborious purification procedures that may take over a month to complete. In 1993, the first study on the adaptation of bacterial artificial chromosome (BAC) technology for viruses was reported (31). Since then, the BAC technology has been indispensable for generating recombinant viruses and has accelerated the studies on viruses with large genomes. The BAC system utilizes *E. coli* and its plasmid F factor. Only one or two F plasmids can exist in an *E. coli* cell; this ensures the stable maintenance of the inserted DNA by reducing the risk of recombination. It can accommodate DNA of about 300 kb, which makes it possible for it to carry large genomes such as that of herpesviruses. Studies have been conducted on MDV-BAC as well. Petherbridge *et al.* reported successful generation of Rispons-BAC, which effectively induced protective immunity against virulent MDV in chickens (44). In Chapter 1, the author also reports the application of the BAC technology for Rispons vector.

The BAC technology provides great advantages for manipulating virus genomes; however, there is a disadvantage: the BAC-derived sequences are unnecessary once the viruses are reconstructed in the eukaryotic cells. To remove these BAC-derived sequences, several methods are used. These include (i) Cre/*loxP* or FLP/*FRT* recombination systems

(Fig. 5A), (ii) cotransfection with repair vector and virus-BAC genome, followed by purification in eukaryotic cells (Fig. 5B), and (iii) self-recombination of duplicated sequences that surround the BAC-derived sequence in the virus (Fig. 5C and 5D). In Cre/*loxP* or FLP/*FRT* recombination methods, the mini-F sequence and the marker gene are flanked by a pair of *loxP* or *FRT* sequences, which are then removed by recombination of a pair of *loxP* or *FRT* sequences. The second method utilizes the original eukaryotic recombination machinery, in which an infectious virus BAC and a homologous sequence fragment are cotransfected into the eukaryotic cell, followed by purification of the BAC-removed virus. The third method also uses original eukaryotic recombination machinery, but it does not require cotransfection of a homologous vector because the virus-BAC is designed with a set of homologous sequences flanking the BAC cassette. The fourth method, the inverted duplicated sequences method, is an improved version of the third one. It utilizes two pairs of inverted sequences, which remove the BAC cassette with low risk of recombination and instability in the *E. coli*. However, all these methods have defects such as the risk of leaving a scar of BAC-derived sequences, the laborious purification required for clonal viruses, and virus instability. Moreover, it is difficult to alter the BAC-excision methods once the virus-BAC is constructed. To overcome these defects and to construct vaccine viruses without a scar of BAC-derived sequences, the

author explores a new BAC-excision method, called removal of inserted BAC after linearization (RIBON), in Chapter 3.

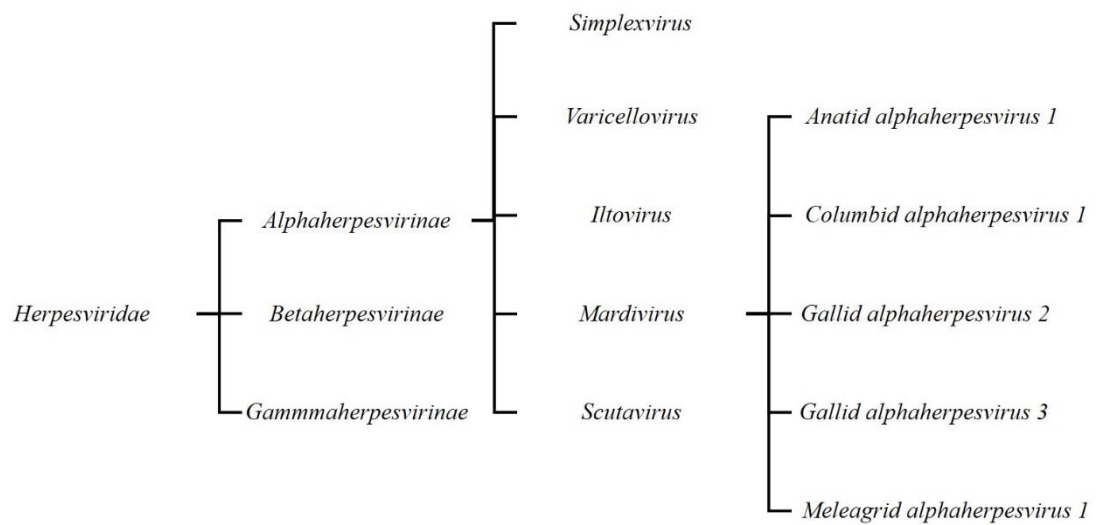


Figure 1. Taxonomy of *Herpesviridae*.

MDV serotypes 1, 2, and 3, also known as *Gallid alphaherpesvirus 2*, *Gallid alphaherpesvirus 3*, and *Meleagrid alphaherpesvirus 1*, respectively, belong to the family *Herpesviridae*, the subfamily *Alphaherpesvirinae*, and the genus *Mardivirus*.

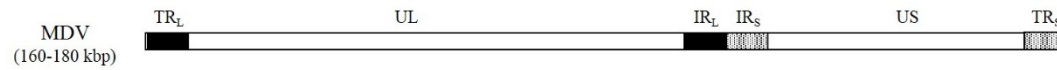


Figure 2. Schema of a typical genome of MDV.

MDV is a class E alphaherpesvirus. Its genome has two unique regions called unique long region (UL) and unique short region (US). UL is flanked by two inverted repeat regions, called the terminal repeat long region (TR_L) and the internal short repeat region (IR_L). Similarly, US is flanked by the terminal short repeat (TR_S) and the internal short repeat (IR_S) regions.



Figure 3. Genetic organization of the Rispens strain.

The genome of the Rispens strain is 178,311 bp long with over 70 genes, similar to other MDVs. The length of the unique long region is 113,490 bp, and it extends from nt 14,477 to nt 127,966. The length of the unique short region is 11,651 bp, and it extends from nt 154,606 to nt 166,256.

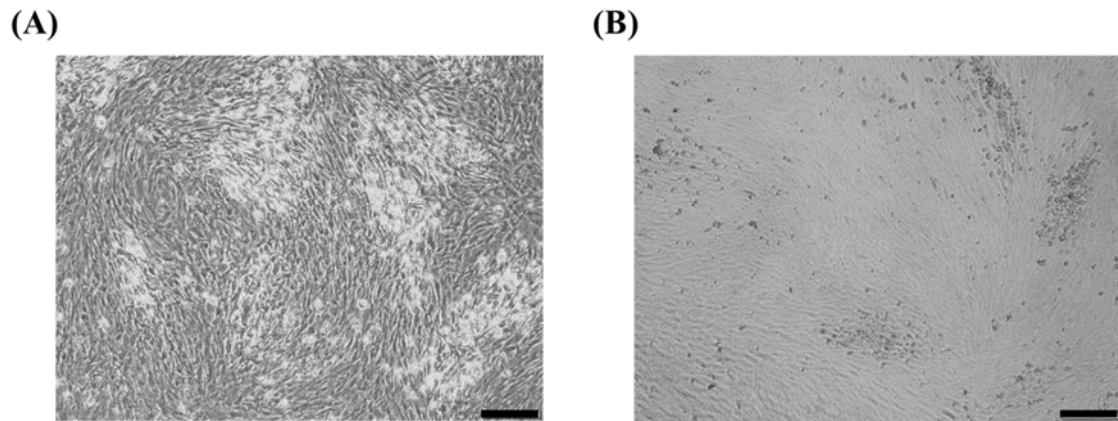


Figure 4. Plaques of HVT or Rispens in CEF.

CEF monolayer was infected with HVT or Rispens strains. Three days after infection, the plaques were observed microscopically. Scale bars, 200 μm . (A) HVT in CEF. (B) Rispens in CEF.

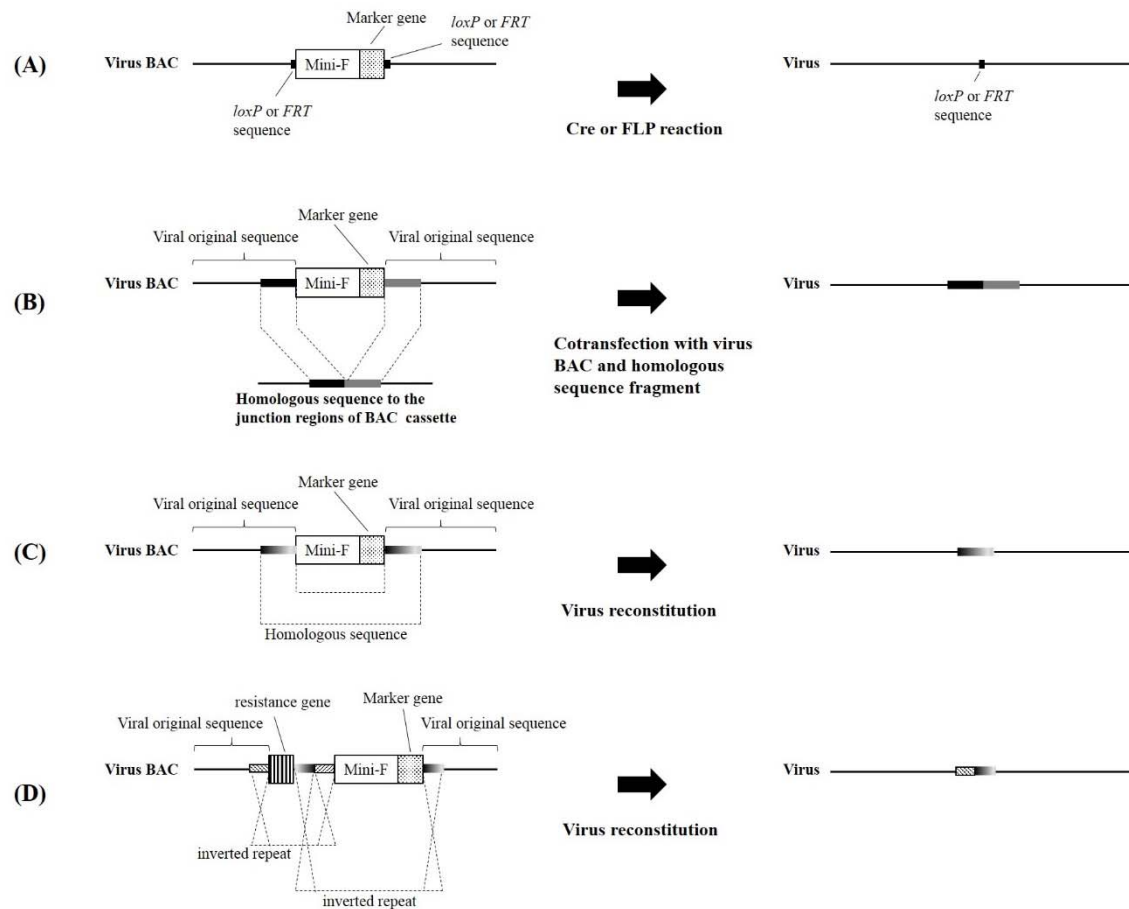


Figure 5. Schema of the four methods to remove mini-F sequences.

(A) Cre/*loxP* or FLP/*FRT* recombination method. Mini-F sequence and marker gene are flanked by a set of *loxP* or *FRT* sequences. The BAC cassette is removed by recombination of a pair of *loxP* or *FRT* sequences with Cre or FLP enzymes. (B) Homologous recombination and purification in eukaryotic cells. Virus-BAC and homologous sequence fragment are cotransfected into eukaryotic cells and the BAC cassette is removed by recombination of the fragment and mini-F-flanking sequences. (C) Duplicated sequences method. Virus BAC is flanked by duplicated sequences and the

BAC cassette is removed by recombination of the duplicated sequences. (D) Inverted duplicated sequences method. The BAC cassette is flanked by two sets of inverted duplicated sequences, which remove the BAC cassette by a series of recombination.

Chapter 1

Development and evaluation of the protective efficacy of novel Marek's disease

virus Rispens vector vaccines against infectious bursal disease

ABSTRACT

Infectious bursal disease (IBD) is a major disease affecting the poultry industry and is caused by infection with IBD virus (IBDV). To develop a novel vaccine to prevent IBD in chickens, recombinant Marek's disease virus Rispens viruses carrying the *VP2* gene of IBDV driven by five different promoters (Rispens/IBD) were constructed using homologous recombination and a bacterial artificial chromosome (BAC). Rispens/IBD driven by the chicken beta-actin (Bac) promoter (Rispens/Bac-IBD), Rous sarcoma virus promoter, or simian virus 40 promoter were administered to 1-day-old SPF chicks, and the protective efficacy against IBDV was evaluated by challenging chicks with virulent IBDV. As a result, Rispens/Bac-IBD showed the best protection (87%). Next, I constructed the virus driven by the Bac-derived Coa5 promoter (Rispens/Coa5-IBD) for a secondary *in vivo* trial using commercial layer chickens since Rispens/Bac-IBD was thought to be genetically unstable. Rispens/Coa5-IBD showed stability *in vitro* and exhibited better antibody production and protection during challenge against virulent IBDV at both 5 (95%) and 7 weeks of age (91%) compared with that of Rispens/Bac-IBD (90% at 5 weeks of age and 84% at 7 weeks of age). Thus, Rispens/Coa5-IBD may be a novel promising vaccine against IBD and virulent Marek's disease.

INTRODUCTION

Herpesviruses have been widely used as vectors for recombinant vaccines because of their ability to infect cells persistently and their capacity for incorporating foreign genes. In the production of poultry vaccines, turkey herpesvirus (HVT) has been commonly used as a vector for recombinant vaccines. Studies have described the use of recombinant HVTs expressing viral antigens, such as Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), avian influenza virus, and infectious laryngotracheitis virus (11)(15)(26)(40). HVT is classified as a type of Marek's disease virus (MDV) serotype 3 and has been used as a vaccine for the prevention of Marek's disease (MD) since the 1970s because of its low pathogenicity in chickens. However, highly virulent strains of MDV emerged in the 1980s, and HVT alone cannot provide clinical protection against such viruses (63). For effective vaccination against highly virulent MDV, administration of Rispens strain with HVT has been shown to be useful (5)(29).

MDV serotype 1 Rispens strain, also called CVI988, has been used as a vaccine for the prevention of MD since the 1970s (46). Because the Rispens strain can provide effective protection against more virulent MDV strains (64), such as the RB1B strain, it is now widely used for vaccination against MD. Vaccines using the Rispens virus as a vector have been investigated as well. Recombinant Rispens viruses expressing antigens of

infectious bronchitis virus, IBDV, or NDV have been reported (30)(55)(67). However, there are still no commercially available Rispons vector vaccines.

Infectious bursal disease (IBD), also known as Gumboro disease, is one of the most important diseases affecting the poultry industry. IBDV, the etiological agent of IBD, causes acute depression, anorexia, and diarrhea in young chickens, particularly from 3 to 6 weeks of age. In infected chickens, IBDV damages B lymphocytes in the bursa of Fabricius, which initially shows severe edema followed by atrophy, and causes immunosuppression (10)(24). From the 1980s, in addition to the classical virulent strain, variant strains that cause high immunosuppression and highly virulent strains that damage the bursa of Fabricius, thymus, spleen, and bone marrow and cause high mortality have emerged (41). This acute and contagious disease has been reported around the world and has caused significant economic losses due to high mortality and immunosuppression. Therefore, control of IBD has become a major concern in the poultry industry, and researchers have focused on the development of an effective vaccine for control of this disease.

Utilization of the Rispons virus as a vector for incorporation of the *VP2* gene from IBDV as an antigen is expected to provide more effective protection against both MD (particularly for highly virulent strains of MDV) and IBD simultaneously. In a previous

report, researchers examined the efficacy of a Rispens vector vaccine expressing IBDV VP2 protein in experimental specific pathogen-free (SPF) chickens (30); however, this is the only type of chicken in which this type of vaccine has been tested to date.

Therefore, in this study, I aimed to explore the development of an effective vaccine for prevention of IBDV using a recombinant Rispens vector expressing the *VP2* gene of IBDV (Rispens/IBD) under various promoters. The protective efficacy of the Rispens/IBD vaccine against IBDV was evaluated in an experimental infection model using both SPF chickens and commercial layer chickens with maternal antibodies against IBDV in order to compare the effects of different promoters on expression of the IBDV *VP2* gene.

MATERIALS AND METHODS

Viruses and cells. Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old SPF chicken embryos (Nissei Bio Company, Yamanashi, Japan) and maintained in a 1:1 mixture of Leibovitz's L-15 (Life Technologies Corp, Tokyo, Japan) and McCoy's 5A Medium (Life Technologies Corp), termed LM, supplemented with 4% calf serum (CS). The parental Rispens strain was obtained from Central Veterinary Institute of Wageningen, the Netherlands. The IBDV standard challenge (STC) strain was used for the IBDV challenge and was obtained from the National Veterinary Services Laboratories, US Department of Agriculture/Animal and Plant Health Inspection Service (Ames, IA, USA). The use of recombinant viruses was confirmed by Ministry of Education, Culture, Sports, Science and Technology (H.24 #2199).

Plasmids. (1) Construction of pUC18-US2-IBD. Two primer pairs (F-EcoRI-SORF3/R-SfiI-US2 and F-SfiI-US2/R-HindIII-US3; Table 1-1) were used for amplification of the US2 flanking region. The resulting 1.1-kbp fragments from polymerase chain reaction (PCR) were cloned into the pUC18 vector (TaKaRa Bio, Shiga, Japan) digested with *EcoRI* and *HindIII*, yielding pUC18-US2-SfiI, which had an *SfiI* recognition site in the middle part of the US2 flanking region. The following were cloned

into the *Sfi*I recognition site of pUC18-US2-*Sfi*I, yielding pUC18-US2-BacIBD: chicken beta-actin (Bac) promoter (nucleotide [nt] 51 to nt 1538 of GenBank #X00182) with added restriction sites for *Sfi*I and *Xba*I obtained by PCR using cellular DNA from CEFs, the IBDV VP2 gene with added restriction sites for *Xba*I and *Sal*I amplified with reverse transcription (RT)-PCR from total genomic RNA from the IBDV STC strain, and simian virus 40 (SV40) polyA signal with added restriction sites for *Sal*I and *Sfi*I obtained by PCR with pBK-CMV (Agilent Technologies, Tokyo, Japan).

The murine cytomegalovirus immediate early 1 (MCMV*ie*1) promoter was synthesized according to the nt 4188 to nt 4731 region of GenBank No. L06816 (Medical & Biological Laboratories Co., Ltd, Aichi, Japan). The MCMV*ie*1 promoter, the Rous sarcoma virus (RSV) promoter of pBK-RSV (Stratagene, Tokyo, Japan), the SV40 promoter of pSI (Promega, Madison, WI, USA), and the Bac-derived Coa5 promoter (nt 352 to nt 622 of GenBank #X00182) of cellular DNA from CEFs, each having the *Sfi*I and *Xba*I recognition sites added by PCR, as well as the *Eco*RI-*Sfi*I fragment (1.1 kbp) of pUC18-US2-BacIBD, were inserted into pUC18-US2-BacIBD digested with *Eco*RI and *Xba*I. Thus, pUC18-US2-MCMV*ie*1IBD, pUC18-US2-RSVIBD, pUC18-US2-SV40IBD, and pUC18-US2-Coa5IBD, respectively, were obtained.

(2) Construction of pUC18-UL44-BAC. Two primer pairs (F-HindIII-UL44/R-*Sfi*I-

UL44 and F-SfiI-UL45/R-EcoRI-UL45; Table 1-1) were used for amplification of the UL44-UL45 flanking region. The resulting 0.9-kbp fragments from PCR were cloned into the pUC18 vector digested with *EcoRI* and *HindIII* to generate pUC18-UL44-SfiI, which had an *SfiI* recognition site in the UL44/UL45 intergenic region. The Coa5 promoter with an added *SfiI* restriction site and the enhanced green fluorescent protein (*eGFP*) gene of pEGFP-1 (TaKaRa Bio) with an added *SalI* restriction site were amplified by PCR with the two primer pairs F-SfiI-LoxP-Coa5/R-eGFP-Coa5 or F-Coa5-eGFP/R-SfiI-LoxP-SalI-eGFP (Table 1-1). These amplicons were mixed and used as a template for subsequent PCR with the F-SfiI-LoxP-Coa5 and R-SfiI-LoxP-SalI-eGFP primers, resulting in a Coa5-eGFP fragment. This process added an *SfiI* recognition site and the loxP sequence to the 5' end of the fragment and an *SalI* recognition site, another loxP sequence, and an *SfiI* recognition site to the 3' end. This fragment was cloned into the T-Cloning site of T-Vector pMD19 (TaKaRa Bio) to generate pCoa5eGFP. The mini-F sequence and the chloramphenicol-resistance gene obtained from pBeloBAC11 (New England BioLabs, Tokyo, Japan) by *SalI* digestion were then cloned into the *SalI* restriction site of pCoa5eGFP, yielding pCoa5eGFP-BAC. Finally, the Coa5 promoter, *eGFP* gene, mini-F sequence, and chloramphenicol-resistance gene were excised from pCoa5eGFP-BAC by *SfiI* digestion and were cloned into the *SfiI* restriction site of

pUC18-UL44-SfiI, yielding pUC18-UL44-BAC.

Construction of Rispens-bacterial artificial chromosome (BAC). The Rispens UL44-UL45 intergenic region was selected as an insertion site for the mini-F cassette. To construct Rispens-BAC, pUC18-UL44-BAC was used as a transfer vector for homologous recombination in CEFs. This plasmid contained *eGFP*, mini-F, and chloramphenicol-resistance genes flanked by 0.9-kbp homologous regions in the *UL44* and *UL45* genes of Rispens. Approximately 2×10^6 CEFs were cotransfected with 1 μ g of pUC18-UL44-BAC and 2 μ g of Rispens genomic DNA by electroporation using Nucleofector II (Lonza, Basel, Switzerland). The cells were then diluted with LM and seeded in 96-well tissue culture plates. Five days after transfection, wells containing eGFP-positive plaque were identified, and cells in the wells were collected by trypsinization and seeded into new 96-well plates.

After three rounds of purification, DNA of recombinant Rispens virus carrying eGFP-BAC (Rispens-BAC) virus was extracted from the infected CEFs as previously described (39). Rispens-BAC genomic DNA was transfected into *Escherichia coli* DH10B cells by electroporation using a Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA) at 1.75 kV, 25 μ F, and 200 Ohm. DH10B cells were then shaken for 1 h at 30°C and seeded

on Luria-Bertani (LB) agar plates containing 20 µg/mL chloramphenicol. After overnight incubation at 30°C, chloramphenicol-resistant colonies were picked and cultured in liquid LB medium. Rispens-BAC DNA from DH10B (pRispens-BAC) was prepared by alkaline lysis of *E. coli*. Band pattern analysis of pRispens-BAC was then performed. pRispens-BAC DNAs were digested with the restriction enzyme *Xho*I (TaKaRa Bio) and separated on 0.8% agarose gel. pRispens-BAC DNA containing the entire genome of Rispens was incubated with Cre recombinase (New England BioLabs) to excise eGFP, mini-F, and chloramphenicol-resistance genes and was then transfected into CEFs using Nucleofector II. Reconstitution of Rispens virus was confirmed by plaque formation.

Growth kinetics of the reconstituted virus. Growth kinetics of reconstituted Rispens virus was compared with parental wild-type Rispens virus. CEFs (4×10^6) in 6-well plates were infected with reconstituted Rispens or parental wild-type Rispens virus at a multiplicity of infection (MOI) of 0.001. The infected cells were overlaid with LM supplemented with 4% CS. The cells were harvested at different times postinfection, and viral titers were determined by plaque assays. The data were obtained from two independent experiments.

Plaque assays. CEFs in 6-well plates were infected with serial 10-fold dilutions of infected cells and overlaid with LM supplemented with 4% CS. Five days after infection, the numbers of the plaques were counted macroscopically.

Construction of Rispens/IBD by homologous recombination. Construction of recombinant Rispens carrying the IBDV *VP2* gene driven by the Bac promoter (Rispens/Bac-IBD) or the MCMV*ie1* promoter (Rispens/MCMV*ie1*-IBD) was conducted by homologous recombination in CEFs. pUC18-US2-BacIBD or pUC18-US2-MCMV*ie1*IBD, which contained the IBDV *VP2* gene under the Bac promoter or the MCMV*ie1* promoter flanked by 1.1-kbp homologous regions surrounding the *US2* gene of Rispens, was used as a transfer plasmid. Approximately 2 µg of Rispens DNA, prepared as previously described (39), and 1 µg of pUC18-US2-BacIBD or pUC18-US2-MCMV*ie1*IBD were transfected into approximately 10⁷ CEFs by electroporation using Nucleofector II. The transfected cells were seeded into 96-well tissue culture plates and incubated for 5–7 days until Rispens plaques became visible. The cells were then trypsinized, transferred equally to two 96-well plates with fresh CEFs, and incubated for 4–6 days. After plaques were visible, screening for plaques expressing IBDV *VP2* protein was conducted by *in situ* immunostaining assays, called black plaque assays, with the

anti-IBDV VP2 monoclonal antibody R63 (54). Wells containing plaques expressing IBDV VP2 protein were identified, and cells from the corresponding wells in the other 96-well plate were diluted and transferred to 96-well plates. After three or four rounds of purification, purified recombinant Rispons/Bac-IBD and Rispons/MCM*Vie1*-IBD were obtained.

Construction of Rispons/IBD using Rispons-BAC. To insert the IBDV *VP2* gene into the US2 region of pRispons-BAC, a dual kanamycin and streptomycin selection marker of the *rpsL*-neo cassette was used (60). A US2-*rpsL*-neo cassette, in which the *rpsL*-neo sequence was flanked by the 50-bp sequences of the US2 insertion region, was synthesized (Medical & Biological Laboratories, Co. Ltd.). First, DH10B cells harboring pRispons-BAC were transformed with pKD46 plasmid for introduction of Red recombinase (12) by electroporation. The transformed cells were used for additional recombination. Then, the US2 region of pRispons-BAC was replaced with the *rpsL*-neo gene cassette, as previously described (27)(60). Briefly, electrocompetent cells of DH10B harboring pRispons-BAC and pKD46, which were induced to synthesize Red recombinase, were transfected with 100 ng of the US2-*rpsL*-neo cassette and seeded on LB agar plates containing 20 µg/mL chloramphenicol, 100 µg/mL ampicillin, and 30

μg/mL kanamycin. After overnight incubation at 30°C, chloramphenicol-, ampicillin-, and kanamycin-resistant colonies obtained from the LB agar plates were checked for gene structures around the US2 region by band pattern analysis and PCR analysis. A DH10B clone harboring an appropriate insertion was then used for insertion of the IBDV *VP2* gene. Electrocompetent cells prepared from the clone were transfected with 100 ng of one of the plasmids (pUC18-US2-RSVIBD or pUC18-US2-SV40IBD) after induction of Red recombinase and seeded on LB agar plates containing 20 μg/mL chloramphenicol and 50 μg/mL streptomycin. The agar plates were incubated overnight at 37°C. DH10B clones carrying an appropriate insert containing the *VP2* gene were identified by PCR analysis and band pattern analysis. Genomic DNAs of pRispen-BAC carrying the IBDV *VP2* gene in the US2 region (pRispen/RSV-IBD and pRispen/SV40-IBD) were extracted from the DH10B clones and transfected into CEFs after incubation with Cre recombinase to excise the *eGFP* gene, mini-F cassette, and chloramphenicol-resistance gene. Finally, two recombinant Rispen viruses carrying the IBDV *VP2* gene in the US2 region were reconstituted in CEFs (Rispen/RSV-IBD and Rispen/SV40-IBD).

Black plaque assay. Expression of IBDV *VP2* protein was confirmed by black plaque assays. Briefly, CEFs were infected with Rispen/IBD and incubated for 3–5 days. The

cells were fixed with a methanol:acetone mixture (1:2) and incubated with the anti-IBDV VP2 monoclonal antibody R63. Next, after incubation with biotinylated anti-mouse IgG antibodies (Vector Laboratories, Burlingame, CA, USA) followed by streptavidin-alkaline phosphatase conjugates (VECTASTAIN ABC-AP kit; Vector Laboratories), plaques expressing VP2 protein were stained by addition of NBT/BCIP solution (Roche Applied Science, Tokyo, Japan).

Western blot analysis. Expression of IBDV VP2 protein was confirmed by western blotting. CEFs were infected with Rispens/IBD or parental wild-type Rispens at an MOI of approximately 0.01. Three days after infection, the cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended with 100 μ L PBS. Then 100 μ L of 2 \times sodium dodecyl sulfate (SDS) sample buffer (130 mM Tris-Cl [pH 6.8], 6% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue) was added, and the cell suspension was boiled for 5 min. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and western blot analysis using R63 anti-IBDV VP2 monoclonal antibodies followed by biotinylated anti-mouse IgG antibodies and streptavidin-alkaline phosphatase conjugates. Protein bound with the R63 monoclonal antibody was visualized by addition of NBT/BCIP solution.

Analysis of the *in vitro* stability of Rispens/IBD. The stability of Rispens/IBD viruses *in vitro* was analyzed by 15 rounds of passage in CEFs. Two clones per Rispens/IBD virus were subcultured every 3–4 days. The expression of IBDV VP2 protein was confirmed every five passages by western blotting and black plaque assays. The genome structures of the viruses were analyzed by PCR. DNA was extracted from CEFs infected with Rispens/IBD or parental wild-type Rispens using Lysis Buffer for PCR (TaKaRa Bio). Extracted DNAs with or without IBDV VP2 sequences were identified by PCR using three primer pairs (Rispens-F/Rispens-R, Rispens-F/VP2-R, and VP2-F/Rispens-R; Table 1-1) for amplification of regions between the IBDV VP2 sequence and the Rispens insertion site sequences.

Efficacy of Rispens/IBD in SPF chicks. The efficacy of Rispens/IBD against classical virulent IBDV was evaluated. Three Rispens/IBD viruses (Rispens/Bac-IBD, Rispens/RSV-IBD, and Rispens/SV40-IBD) were used in this trial. One-day-old SPF white leghorn chicks (Nissei Bio Company) were divided into five groups; three groups were inoculated subcutaneously with a single dose of one of the Rispens/IBD viruses. The remaining two groups were left unvaccinated as controls.

Blood was collected from the chickens each week under 7 weeks of age for evaluation

of humoral immunity against IBDV. The amount of serum anti-IBDV antibodies was measured using an IBDV ELISA kit (FlockChek IBD; Idexx Laboratories, Tokyo, Japan). At 7 weeks of age, all chickens except one unvaccinated group (Group 1) were challenged orally with 10^3 of the 50% egg infectious dose (EID₅₀) of the virulent IBDV STC strain. The chickens were checked daily for clinical signs associated with IBD. Seven days after the challenge, chicks were euthanized and necropsied to identify grossly observable bursal lesions, such as edema, discoloration, atrophy, hemorrhage, and yellow or gelatinous exudates. The presence or absence of bursal lesions was recorded for each animal. Body and bursa weights were measured for calculation of the B/B index, defined as the ratio between the bursa weight and the body weight of challenged birds divided by the same ratio of non-challenged birds.

Construction of Rispens/Coa5-IBD. To insert the IBDV VP2 gene driven by Coa5 promoter into the US2 region of pRispens-BAC, DH10B cells harboring pKD46 and pRispens-BAC with the rpsL-neo gene cassette in its US2 region were transfected with pUC18-US2-Coa5IBD, and screening and reconstitution of Rispens/Coa5-IBD were conducted as described above. Expression of the VP2 protein by Rispens/Coa5-IBD was confirmed by western blotting and black plaque assays as described above. *In vitro*

genomic stability was also evaluated as described above.

Efficacy of Rispens/IBD in commercial chicks. In this trial, the efficacy of the Bac-derived Coa5 promoter in chickens possessing maternal antibodies was assessed. Rispens/Bac-IBD was also used as a positive control. Approximately 160 one-day-old commercial layer chicks (Kanagawa Youkeiren, Kanagawa, Japan) with maternal antibodies were divided into four groups, each containing approximately 40 chicks. Chicks in Groups 3 and 4 were inoculated subcutaneously with a single dose of Rispens/Bac-IBD or Rispens/Coa5-IBD, respectively. At 5 weeks after vaccination, half of the chickens in Groups 2–4 were challenged orally with 10^3 EID₅₀ of the virulent IBDV STC strain; the other chickens remained unchallenged, but were then challenged orally with 10^3 EID₅₀ of the IBDV STC strain at 7 weeks after vaccination. For evaluation of humoral immunity against IBDV, blood was collected from the chickens each week until 7 weeks of age, and the amount of serum anti-IBDV antibodies was measured using the IBDV ELISA kit (Idexx Laboratories). After the challenge, the chickens were checked daily for clinical signs associated with IBD. Seven days after each challenge, the challenged chickens and chickens in Group 1 (unvaccinated, unchallenged chickens) were euthanized and necropsied to identify grossly observable bursal lesions. The

presence or absence of bursal lesions was recorded, and the B/B index was calculated by measuring the body and bursa weights.

Ethical statement. All animal experiments were approved by the ethics committee of Ceva Animal Health. All animal procedures strictly followed the company's guidelines for animal care and use.

RESULTS

Construction of Rispens-BAC. Rispens-BAC was successfully constructed. The UL44/UL45 intergenic region was used as an insertion site for the mini-F cassette (Fig. 1-1A). Two Rispens-BAC clones were obtained, and one of the clones (clone #1) showed the expected restriction enzyme digestion pattern (Fig. 1-1B). Reconstituted Rispens showed similar cytopathic effects as wild-type Rispens in CEFs after excision of *eGFP* and the mini-F cassette by Cre recombinase treatment. Further analysis of reconstituted Rispens was conducted by comparing the growth kinetics with those of wild-type Rispens (Fig. 1-1C). Reconstituted Rispens exhibited a growth rate similar to that of the wild-type Rispens, suggesting that the *in vitro* growth of reconstituted Rispens was equivalent to that of the wild-type virus.

Construction of Rispens/IBD. The US2 region was selected as an insertion site for the IBDV *VP2* gene (Fig. 1-1A). First, Rispens/Bac-IBD and Rispens/MCMV*ie1*-IBD were constructed by homologous recombination in CEFs. Although Rispens/Bac-IBD and Rispens/MCMV*ie1*-IBD were successfully constructed, the recombination rate of the IBDV *VP2* gene cassette into the US2 insertion site was only less than 5%, and pure clones of the recombinant viruses were only obtained after up to several months of

rigorous purification. On the other hand, other two Rispens/IBD viruses (Rispens/RSV-IBD and Rispens/SV40-IBD) were successfully constructed using Rispens-BAC in only about 10 days with less complicated procedures.

***In vitro* characteristics of Rispens/IBD.** IBDV VP2 expression in CEFs infected with the four Rispens/IBD viruses (Rispens/Bac-IBD, Rispens/MCMVie1-IBD, Rispens/RSV-IBD, and Rispens/SV40-IBD) was analyzed (Figs. 1-2A and 2B). Protein bands of 40 kDa, the expected size of the VP2 protein, were observed for all of the four Rispens/IBD viruses in western blot analysis (Fig. 1-2B). Rispens/Bac-IBD appeared to induce the highest amount of VP2 protein. Rispens/MCMVie1-IBD also induced strong expression; however, Rispens/RSV-IBD and Rispens/SV40-IBD induced slightly lower expression than the other viruses.

Two clones for each of the four Rispens/IBD viruses were subcultured in CEFs 15 times, and the *in vitro* stability was analyzed by western blotting, black plaque assays, and PCR analysis. All viruses except for one clone of Rispens/Bac-IBD and both clones of Rispens/MCMVie1-IBD passed the tests (data not shown), and stability was confirmed in cultured CEFs. The one clone of Rispens/Bac-IBD was found to carry a deletion in the Bac promoter-IBDV VP2 gene at the 15th passage. The two clones of

Rispens/MCMVie1-IBD had similar deletions at as early as the fifth passage. In addition, while plaques of Rispens/Bac-IBD, Rispens/RSV-IBD, and Rispens/SV40-IBD could be observed as early as 3–4 days after infection, Rispens/MCMVie1-IBD grew more slowly, and plaques were not observed until 6–7 days after infection. Because of the instability and slow growth of Rispens/MCMVie1-IBD in CEFs, no further analysis of this virus was performed.

Efficacy trial of Rispens/IBD in SPF chickens. In the first trial, SPF chickens were challenged at 7 weeks postvaccination. This condition was set in order to briefly screen the protection capacities of the developed viruses. Three Rispens/IBD viruses (Rispens/Bac-IBD, Rispens/RSV-IBD, and Rispens/SV40-IBD) were inoculated into 1-day-old SPF chickens, and the efficacy of the vaccine against IBDV challenge was evaluated. Because one clone of Rispens/Bac-IBD was found to be unstable *in vitro*, I used the other clone, which was stable after *in vitro* passage. Anti-IBDV antibody titers, as quantified using an IBDV ELISA kit, revealed that all of the vaccinated groups developed humoral immunity against IBDV from 3 weeks of age (Fig. 1-3). The titers in Rispens/Bac-IBD-vaccinated chickens were consistently higher than those in Rispens/RSV-IBD- or Rispens/SV40-IBD-vaccinated chickens. Consistent with this

result, the protection provided by Rispens/Bac-IBD was 87% (13/15), which was higher than the protection provided by Rispens/RSV-IBD and Rispens/SV40-IBD (53% and 33%, respectively; Table 1-2). The average B/B Index of the chickens vaccinated with Rispens/Bac-IBD was 0.95, suggesting that there was no significant atrophy in the bursa.

However, since genetic instability of Rispens/Bac-IBD was suspected from the *in vitro* study, peripheral lymphocytes from three of the vaccinated chickens in this trial were isolated and expression of VP2 in re-isolated viruses was examined using black plaque assays (data not shown). Although coculture of the lymphocytes with CEFs led to formation of plaques, VP2 expression was observed only in some of the plaques (data not shown).

Construction and *in vitro* evaluation of Rispens/Coa5-IBD. Although Rispens/Bac-IBD showed strong VP2 expression and good protection rates *in vivo*, genetic instability was suspected. Thus, Rispens/Coa5-IBD was constructed using the Rispens-BAC system. The Coa5 promoter consists of the core region of the Bac promoter. Rispens/Coa5-IBD showed intermediate expression of the VP2 protein *in vitro* compared with the other four viruses (Figs. 1-2A and 2B). Rispens/Coa5-IBD passed an *in vitro* serial passage test, in which the genetic stability of the constructed virus was confirmed by western blotting,

black plaque assays, and PCR analysis (data not shown). In addition, plaques of Rispens/Coa5-IBD could be observed 3–4 days after infection, similar to Rispens/Bac-IBD, Rispens/RSV-IBD, and Rispens/SV40-IBD.

Efficacy trial of Rispens/Coa5-IBD in commercial chickens. In the second *in vivo* trial, Rispens/Bac-IBD and Rispens/Coa5-IBD were compared in commercial chickens with maternal antibodies. After challenge at 5 weeks of age, both groups vaccinated with Rispens/IBD exhibited excellent protection (90% for Rispens/Bac-IBD and 95% for Rispens/Coa5-IBD), while all of the chickens in the nonimmunized, challenged positive control (NICC; Group 2) developed gross bursal lesions typical of IBD (Table 1-3). In this group, because NICC chickens showed either swelling (increased B/B index) or atrophy (decreased B/B index) of the bursa, the average B/B index appeared to be similar to those of the other groups. In the 7-week-challenge groups, most of the chickens in the NICC group showed atrophy of the bursa, leading to an apparent decrease in the average B/B index. Similarly, excellent protection was observed after challenge at 7 weeks of age (Table 1-4). Both groups vaccinated with Rispens/IBD again exhibited excellent protection (84% for Rispens/Bac-IBD and 91% for Rispens/Coa5-IBD), while all of the chickens in the NICC (Group 2) developed gross IBD bursal lesions (Table 1-4).

Rispens/Coa5-IBD provided better protection than Rispens/Bac-IBD after challenge at both 5 and 7 weeks of age. Consistent with this result, anti-IBDV antibody titers in Rispens/Coa5-IBD-vaccinated chickens were higher than those in Rispens/Bac-IBD vaccinated chickens between 5 and 7 weeks of age (Fig. 1-4).

DISCUSSION

In this study, I developed Rispens/IBD viruses driven by several different promoters, and their phenotypes and efficacy were evaluated *in vitro* and *in vivo*. Combination of an inserted antigen gene, a promoter, and an insertion site has been shown to affect the protective efficacy of vaccines produced using recombinant viruses (16)(58). The CMV promoter is commonly used for HVT (16), whereas the MDV1 glycoprotein B (gB) promoter and MDV1 bidirectional promoter are reported to be effective for MDV1 (55)(68). In recombinant Rispens, the inserted antigen of the NDV fusion protein driven by the MDV1 gB promoter was shown to induce more effective immune responses against NDV than the SV40 promoter (55). However, information regarding promoter selection for expression of antigen genes in recombinant Rispens vaccines is still limited. Therefore, I compared the Bac, MCMV*ie1*, RSV, SV40, and Coa5 promoters to drive the expression of the *VP2* gene of IBDV. The Bac and MCMV*ie1* promoters are known to induce strong gene expression, whereas the RSV and SV40 promoters induce lower gene expression. The length of the Bac promoter is approximately 1,500 bp, whereas the lengths of the MCMV*ie1*, RSV, and SV40 promoters are between 500 and 700 bp. The Coa5 promoter, which is 274 bp in length and consists of the core region of the Bac promoter, was previously developed by our laboratory for improvement over the Bac

promoter (unpublished data), because during insertion of foreign genes into viruses, the insert length and virus stability have been reported to be negatively correlated in several viruses (2)(3)(7). The expression driven by the Coa5 promoter is high, but slightly lower than that of the Bac promoter (unpublished data).

Among the promoters investigated in this study, Rispens/IBD carrying the *MCMVie1* promoter was noticeably slower in developing visible plaques than other Rispens/IBD viruses. No plaque formation with this virus was observed during passages 1–3 in the cells (data not shown). It was reported that cytopathic effects were found to develop as the duration of culture and number of passages were increased, and that plaques gradually became visible with repeated passaging (8). Rispens/IBD with the *MCMVie1* promoter may have become adapted to CEFs by repeated passaging in the cells, finally developing visible plaques. Moreover, one report showed that the *MCMVie1* promoter inhibits plaque formation for MDV1 (35). My results also suggested that the *MCMVie1* promoter had low compatibility with MDV1.

In the first trial, vaccination with the recombinant viruses having promoters that induced lower gene expression (i.e., Rispens/RSV-IBD and Rispens/SV40-IBD) resulted in reduced antibody production and low protection against IBDV challenge in chickens. On the other hand, vaccination using Rispens/Bac-IBD, which exhibited the highest

expression of VP2 protein among the Rispens/IBD viruses, provided superior protection. This superior immune response of Rispens/Bac-IBD was probably elicited by high expression of VP2 protein. However, one of two Rispens/Bac-IBD clones lost a portion of the inserted sequence upon *in vitro* passaging, and potential instability was a concern for the Rispens/Bac-IBD clone, which had passed the *in vitro* stability test. In my *in vivo* experiment, instability of Rispen/Bac-IBD was again suspected by isolation of the virus from leukocytes. Based on these results, I concluded that Rispens/Bac-IBD was unstable and decided to use Rispens/Coa5-IBD, which applies the shorter promoter derived from the Bac promoter, in the second trial.

In the second trial, chickens with maternal antibodies against IBDV were used. In such chickens, vaccination with traditional live attenuated IBDV vaccines could be very complicated, particularly in terms of timing of vaccination, because the administered viruses may be neutralized by maternal antibodies and because the levels of maternal antibodies in a flock are not uniform (10). In many cases, more than one vaccination may be necessary to provide sufficient protection in the entire flock. Depending on the level of maternal antibodies, chickens may be protected against virulent IBDV until 3–4 weeks of age. In this study, single vaccination at 1 day of age with Rispens/Coa5-IBD provided excellent protection against IBDV challenge at as early as 5 weeks of age in commercial

layer chickens with maternal antibodies against IBDV. The protection rates of Rispens/Coa5-IBD in the 5-week trial and 7-week trial using commercial chickens were 95% and 91%, respectively. In a previous report, in which recombinant Rispens expressing VP2 protein under the control of the human cytomegalovirus promoter in its US10 region was used in 32-day-old SPF chickens, the protection rate reached up to 80%. Although Rispens/Coa5-IBD was not evaluated using SPF chickens, Rispens/Bac-IBD showed a protection ratio of 87% in the first trial using SPF chickens. These results indicated that the Rispens/IBD vaccines developed in this study may induce more effective immunity than that achieved in the previous report, even under the influence of maternal antibodies. Furthermore, anti-IBDV ELISA titers in chickens vaccinated with Rispens/Coa5-IBD started to increase between 4 and 5 weeks of age, after maternal antibodies declined by about 4 weeks of age. My results showed that vaccination with Rispens/Coa5-IBD could provide protection against IBD at as early as 5 weeks of age for replacement of the protection provided by maternal antibodies. Because chickens are most susceptible to IBDV at 3–6 weeks of age, a combination of maternal antibodies and vaccines is required to protect chickens during this period. My study demonstrated that Rispens/Coa5-IBD could protect chickens at 5–7 weeks of age. In addition, the lowest ELISA titer of chickens inoculated with Rispens/Coa5-IBD at 4 weeks of age exceeded

the ELISA titers for Rispens/Bac-IBD at 5 weeks of age, demonstrating 90% protection. These results indicated that the Rispens/Coa5-IBD vaccines developed in this study could provide effective protection against IBD in chickens with maternal antibodies during the susceptible period. Additional trials using younger chickens are needed to test this hypothesis.

The Coa5 promoter originates from the Bac promoter and is shorter in length than the Bac promoter. Therefore, I expected that Rispens/Coa5-IBD would exhibit improved stability over the Bac promoter. From my analysis, I found that Rispens/Coa5-IBD expressed less VP2 protein than Rispens/Bac-IBD *in vitro*; however, Rispens/Coa5-IBD conferred better protection against IBDV challenge in chickens *in vivo*. Based on the results obtained in first trial, it is possible that some of the Rispens/Bac-IBD virus in the vaccinated chickens stopped producing VP2 protein due to the instability of the virus. Another possible explanation is that Rispens/Coa5-IBD may have superior proliferative potential *in vivo* than Rispens/Bac-IBD because the inserted sequence was shorter and could have had less effect on viral replication. In any case, the elevated serum anti-IBDV antibody levels observed in Rispens/Coa5-IBD-inoculated chickens indicated improved immune stimulation *in vivo*. Therefore, it should be emphasized that both the *in vitro* expression levels of target genes and the genetic stability of the virus may be important

for inducing strong immune responses in animals.

In this study, I developed several Rispens/IBD viruses using the BAC system. Use of Rispens virus as a vector to express foreign antigens provides a novel option for multivalent vaccination in the poultry industry. However, genetic engineering of the Rispens strain using the traditional homologous recombination technique is a time-consuming and labor-intensive process due to low recombination efficiency; indeed, it can take up to several months to complete the screening process. On the other hand, the use of the Rispens-BAC system constructed in this study accelerated the entire procedure and provided high recombination efficiency. Application of the BAC system is considered to be an attractive choice for the genetic engineering of viruses that are difficult to manipulate with traditional homologous recombination techniques, such as the Rispens strain.

In conclusion, I developed a novel promising vaccine virus for chicken IBD using Rispens as a vector virus. Rispens/Coa5-IBD showed excellent protection in commercial chickens against IBD challenge. I found that the Coa5 promoter was the most suitable promoter for the Rispens/IBD virus from the five promoters evaluated in this study. However, promoter analyses were only performed with the *VP2* gene of IBDV and with the US2 region in this study. Insertion of other antigen genes from other pathogens into

several sites will provide further insights into the effective development of polyvalent vaccines using Rispens virus. Additionally, the Rispens-BAC method adopted in this study to prepare recombinant vaccine viruses seemed to be superior to traditional homologous recombination in terms of high recombination efficacy. Simultaneous protection efficacy of Rispens/IBD against highly virulent MDV as well as compatibility with other vaccines and utility of the Rispens-BAC system for polyvalent vaccine development should be further evaluated.

Table 1-1. Primers used for generating plasmids and for *in vitro* stability analysis

Primer	Sequence (5'-3')
F-EcoRI-SORF3	GCGAATTCTGCCCCGCTAAGGAC
R-SfiI-US2	GCCACCAGATGGAAGTGGGGCCAATAAGGCCGGTATGCCGTGGGCC
F-SfiI-US2	GGCCCACGGCATAACGGCCTTATTGGCCCCAGTTCCATCTGGTGGC
R-HindIII-US3	GCAAGCTTTTTACATGCTGCCCAA
F-HindIII-UL44	CCAAGCTTACATGATGAAGCGCAGCCTAACAC
R-SfiI-UL44	ATTCATAGAAACGATATGGCCAATAAGGCCTTAAAAGGCTAAGAAATGCG
F-SfiI-UL45	CATTTCTTAGCCTTTTAAGGCCTTATTGGCCATATCGTTTCTATGAATATTAG
R-EcoRI-UL45	ACGAATTCTGTTTATTTTTGTGTTTATTTTC
F-SfiI-LoxP-Coa5	GGCCTTATTGGCCATAACTTCGTATAGCATACATTATACGAAGTTATTATTTTGTGCAGCGATGGGG
R-eGFP-Coa5	GCCCTTGCTCACCATACGCGGTCAGTCAGAGCC
F-Coa5-eGFP	GGCTCTGACTGACCGCGTATGGTGAGCAAGGGCGAGGAGCT
R-SfiI-LoxP-SalI-eGFP	GGCCAATAAGGCCATAACTTCGTATAATGTATGCTATACGAAGTTATGTCTGACAGATACATTGATGAGTTTGGAC
Rispens-F	GTGGGGACCCGAGGATTTTG
Rispens-R	CAGGATGATGTGGGTGTGC
VP2-F	TGAACTAGCAAAGAACCTGG
VP2-R	GCCAGGGAATCCAGGGAAAAAGAC

Table 1-2. Protection of Rispens/IBD against IBDV challenge in 7-week-old SPF chickens.

Group number	Viruses used for vaccination	Number of chicks	B/B Index^a (SD)	Number of dead chickens after challenge	Number of birds having bursal lesions/total animal number	% Protection
1	NINC ^b	13	1.00 (0.24)	0	0/13	Not applicable
2	NICC ^c	13	0.47 (0.29)	0	13/13	0%
3	Rispens/Bac-IBD	15	0.95 (0.26)	0	2/15	87%
4	Rispens/RSV-IBD	15	0.77 (0.19)	0	7/15	53%
5	Rispens/SV40-IBD	15	0.59 (0.18)	0	10/15	33%

^aB/B Index = bursa weight to body weight ratio (BB ratio) of infected birds/BB ratio of NINC

^bNINC = nonimmunized, unchallenged negative controls

^cNICC = nonimmunized, challenged positive controls

Table 1-3. Protection of Rispens/IBD against IBDV challenge in 5-week-old commercial layer chickens.

Group number	Viruses used for vaccination	Number of chicks	B/B Index^a (SD)	Number of dead chickens after challenge	Number of birds having bursal lesions/total animal number	% Protection
1	NINC ^b	20	1.00 (0.24)	0	0/20	Not applicable
2	NICC ^c	22	1.08 (0.38)	3	22/22	0%
3	Rispens/Bac-IBD	20	1.09 (0.24)	0	2/20	90%
4	Rispens/Coa5-IBD	22	1.08 (0.18)	0	1/22	95%

^aB/B Index = bursa weight to body weight ratio (BB ratio) of infected birds/BB ratio of NINC

^bNINC = nonimmunized, unchallenged negative controls

^cNICC = nonimmunized, challenged positive controls

Table 1-4. Protection of Rispens/IBD against IBDV challenge in 7-week-old commercial layer chickens.

Group number	Viruses used for vaccination	Number of chicks	B/B Index^a (SD)	Number of dead chickens after challenge	Number of birds having bursal lesions/total animal number	% Protection
1	NINC ^b	20	1.00 (0.14)	0	0/20	Not applicable
2	NICC ^c	19	0.55 (0.24)	5	19/19	0%
3	Rispens/Bac-IBD	19	0.96 (0.28)	0	3/19	84%
4	Rispens/Coa5-IBD	22	0.96 (0.15)	1	2/22	91%

^aB/B Index = bursa weight to body weight ratio (BB ratio) of infected birds/BB ratio of NINC

^bNINC = nonimmunized, unchallenged negative controls

^cNICC = nonimmunized, challenged positive controls

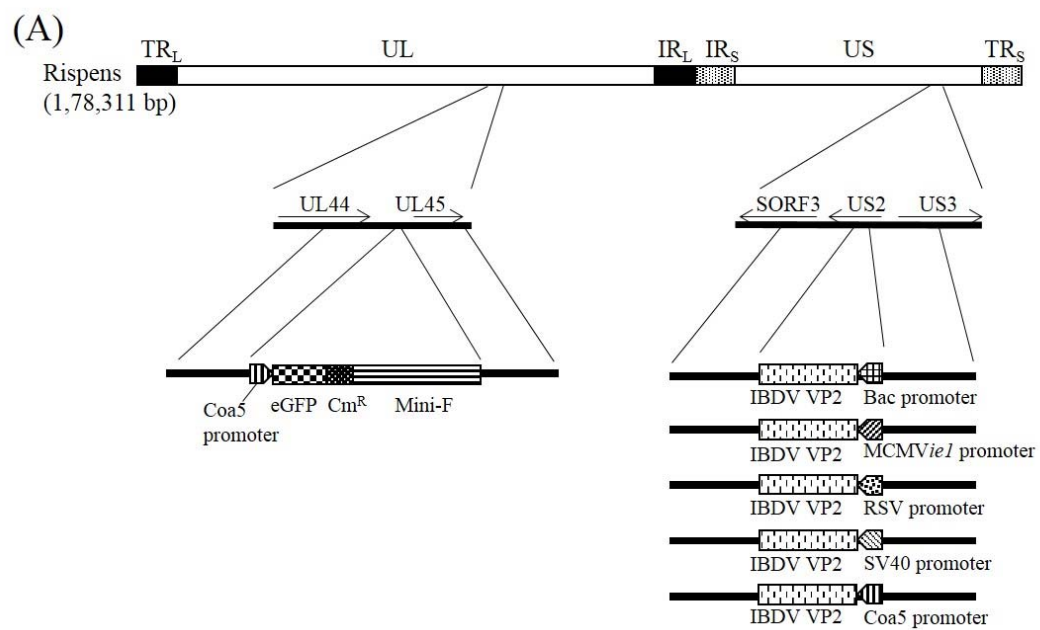


Figure 1-1A

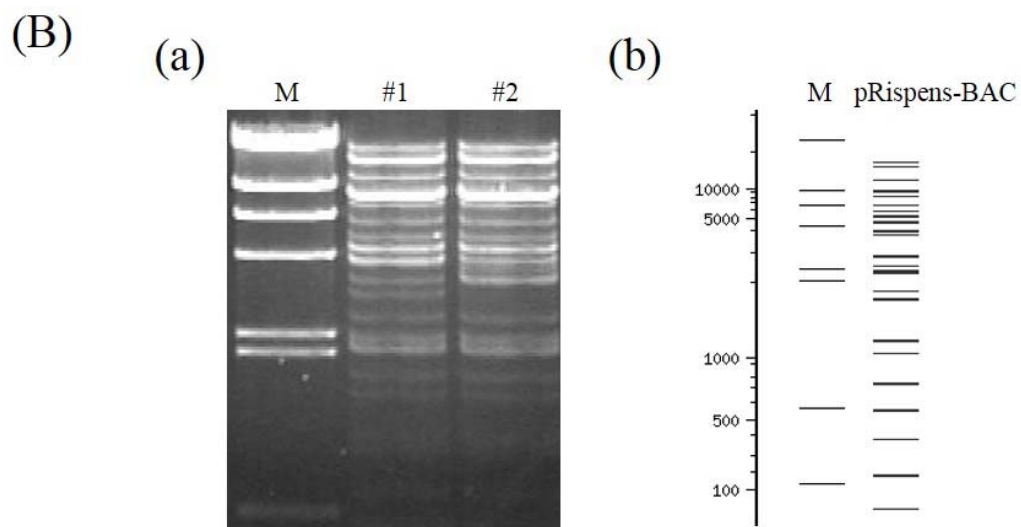


Figure 1-1B

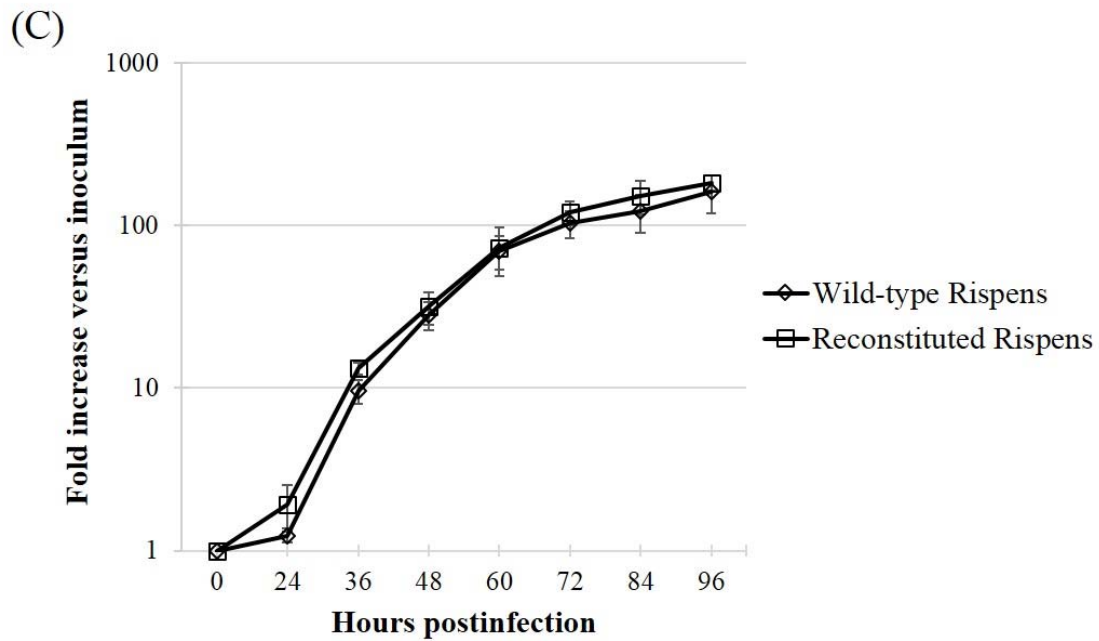


Figure 1-1C

Figure 1-1. Characteristics of Rispens-BAC.

(A) Schematic diagram of the Rispens strain and inserted genes. The corresponding regions of viral DNA are shown as lines. The *eGFP* gene driven by the Coa5 promoter, the chloramphenicol-resistance gene (Cm^R), and the mini-F sequence were inserted into the UL44/UL45 intergenic region. The IBDV *VP2* gene driven by the Bac, MCMVie1, RSV, SV40, or Coa5 promoters was inserted into the US2 region. (B) Comparison of the Rispens band pattern digested with *XhoI*. (a) Band pattern of two clones of pRispens-BAC. M: Lambda DNA-*HindIII* digest marker. (b) Expected band pattern of pRispens-BAC. The sequence of pRispens-BAC was submitted to NEBcutter V2.0 to be digested

with *HindIII*, and the virtual band pattern was generated. (C) Comparison of the growth kinetics of wild-type Rispens and reconstituted Rispens. Monolayers of CEFs were infected with wild-type Rispens or reconstituted Rispens. Infected cells were harvested at different time points after infection, and the titers were determined using plaque assays. Two independent infections were performed, and averages are shown.

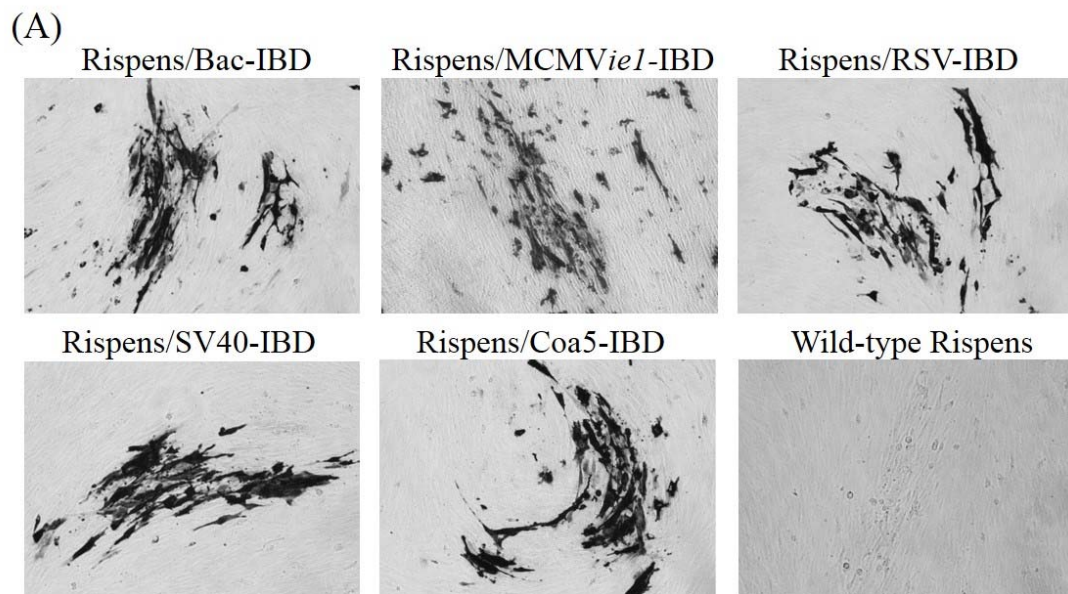


Figure 1-2A

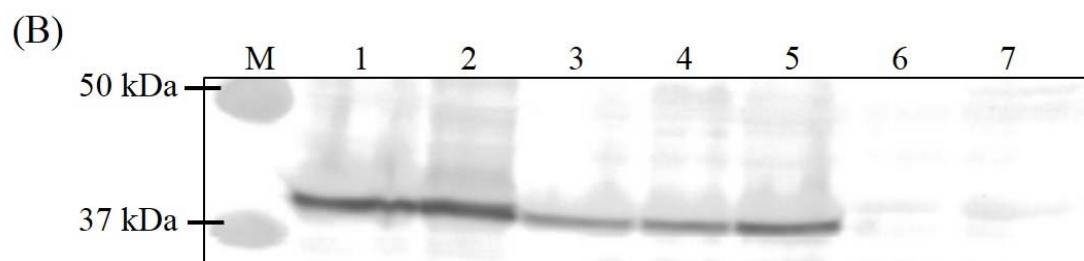


Figure 1-2B

Figure 1-2. Characteristics of Rispens/IBD.

(A) Black plaque assays of Rispens viruses. Monolayers of CEFs were infected with Rispens/Bac-IBD, Rispens/MCMVie1-IBD, Rispens/RSV-IBD, RispensSV40-IBD, Rispens/Coa5-IBD, or wild-type Rispens. Three days after infection, the cells were subjected to black plaque assays for detection of IBDV VP2 protein. (B) Comparison of

IBDV VP2 expression by recombinant Rispens viruses. CEFs were infected with Rispens/Bac-IBD (lane 1), Rispens/MCM*Vie1*-IBD (lane 2), Rispens/RSV-IBD (lane 3), RispensSV40-IBD (lane 4), Rispens/Coa5-IBD (lane 5), or wild-type Rispens (lane 6) at an MOI of 0.01. Three days after infection, the cells were harvested and subjected to western blot analysis for detection of IBDV VP2 protein. M: marker. Lane 7: uninfected CEF control.

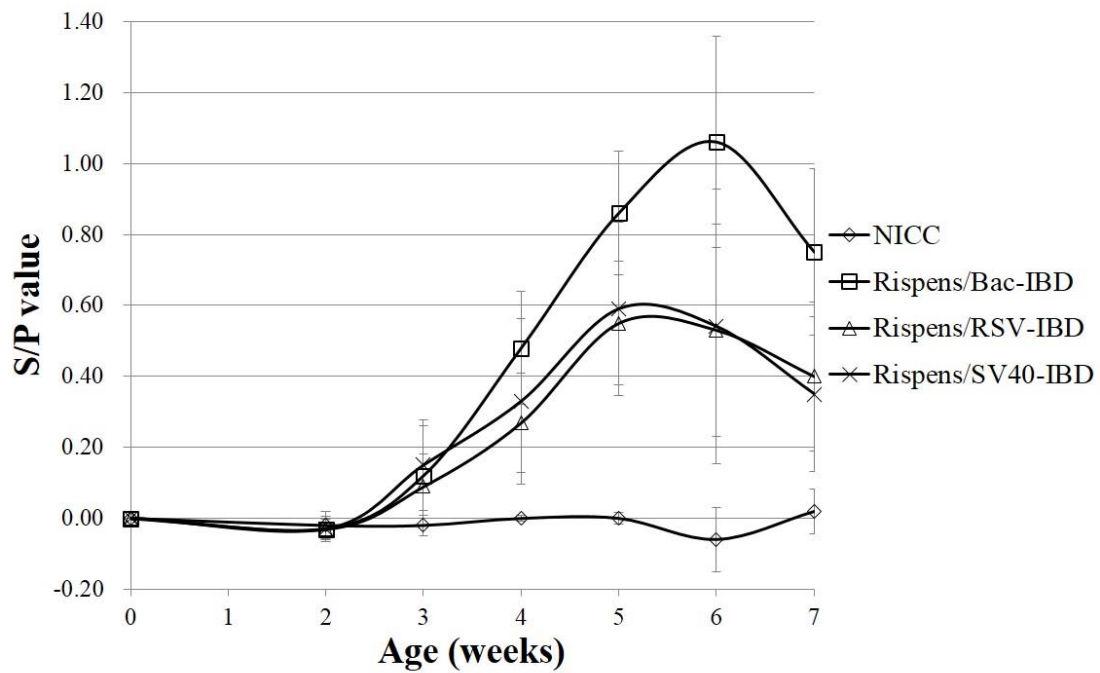


Figure 1-3. IBDV ELISA titers in SPF chickens vaccinated with Rispens/IBD.

One-day-old SPF chicks were vaccinated with a single dose of Rispens/IBD. Blood was collected from the chickens each week until 7 weeks of age and used for evaluation of humoral immunity against IBDV. The amount of serum anti-IBDV antibodies was measured using an IBDV ELISA kit. Sample-to-positive (S/P) values were calculated, with the cutoff value of seroconversion set at 0.2. NICC = nonimmunized, challenged positive control.

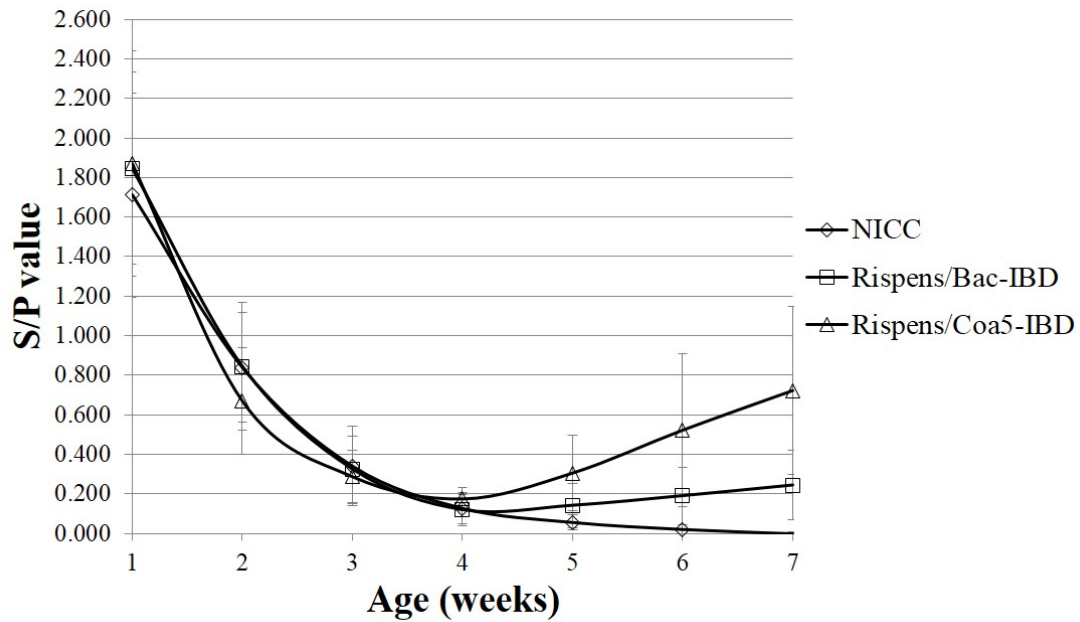


Figure 1-4. IBDV ELISA titers in commercial chicks vaccinated with Rispens/IBD.

One-day-old commercial chicks were vaccinated with a single dose of Rispens/IBD.

Blood was collected from the chickens each week until 7 weeks of age and used for

evaluation of humoral immunity against IBDV. The amount of serum anti-IBDV

antibodies was measured using an IBDV ELISA kit. Sample-to-positive (S/P) values were

calculated, with the cutoff value of seroconversion set at 0.2. NICC = nonimmunized,

challenged positive control.

Chapter 2

**Combination of two Marek's disease virus vectors shows effective vaccination
against Marek's disease, infectious bursal disease, and Newcastle disease**

ABSTRACT

Herpesvirus of turkeys (HVT) is a widely used vector for poultry vaccines. However, different HVTs expressing different foreign antigens cannot always be used simultaneously because of the risk of recombination and interference. In this chapter, I inoculated a mixture of an HVT expressing the antigen of Newcastle disease virus (NDV; HVT/ND) and Marek's disease virus (MDV) serotype 1 Rispens virus expressing the antigen of infectious bursal disease virus (IBD; Rispens/IBD) into chickens. This mixture showed 94%, 100%, or 94% protection against MDV, IBDV, or NDV challenge, respectively. In conclusion, the combination of Rispens/IBD and HVT/ND is effective for vaccination against MDV, IBDV, and NDV without significant interference.

INTRODUCTION

Control of infectious diseases is one of the most important concerns in the poultry industry. In particular, Newcastle disease (ND), Marek's disease (MD), and infectious bursal disease (IBD) are widespread and have caused significant economic losses worldwide because of their high transmission and mortality rates. Therefore, many vaccines have been developed and investigated to control these diseases.

Recombinant vaccines, which are established from viruses harboring inserted foreign antigen genes in their genomes, were first reported in 1982 and have been widely investigated since (32)(43). Herpesvirus of turkeys (HVT), Newcastle disease virus (NDV), adenovirus, and fowlpox virus have been widely used as vectors of chicken recombinant vaccines. Among these viruses, HVT, which belongs to Marek's disease virus (MDV) serotype 3 in the subfamily *Alphaherpesvirinae*, has been most commonly used owing to its capacity to evade interference by maternally derived antibodies, its good safety, and its ability for persistent infection and long duration of immunity. Recombinant HVTs carrying antigen genes of NDV (HVT/ND) or infectious bursal disease virus (IBDV, HVT/IBD) have been developed and are now commercially available (11)(14). These recombinant HVTs show excellent protection levels and are widely used; however, they cannot be used simultaneously because of the risk of interference on protective efficacy.

A previous study showed that simultaneous inoculation of HVT/ND and HVT/IBD reduced the protection level against each inserted antigen compared to single inoculation (50). In contrast, no interference of the protection against inserted antigens was reported for recombinant HVTs expressing the antigen protein of avian influenza or NDV in chickens (45). Thus, although there are several reports on the simultaneous inoculation of two closely related but distinct HVTs, there are no detailed studies examining the effects of simultaneous infection.

Studies on the simultaneous infection or coinfection of two related or mutant viruses of *Alphaherpesvirinae* into animals have been performed using herpes simplex virus (42), pseudorabies virus (17)(20), and bovine herpesvirus (49). Indeed, in these studies, recombination of two viruses was reported in the infected animals. A similar study was also performed with MDV serotype 1 (MDV1), which belongs to the same genus as HVT, *Mardivirus* (21). Coinfection of two distinct viruses was observed in the feather follicle epithelial cells of chickens inoculated simultaneously with two RB-1B strains of MDV1 carrying different foreign genes in their genomes; however, most of the feather follicle epithelial cells were infected with only one or the other virus (21). This result indicated that recombination of MDV1 viruses could occur in chickens, and that inhibition of viral superinfection occurred at the cellular level in the vaccinated chickens. Similar results on

viral interference were also reported for rats inoculated with two pseudorabies virus recombinants (25). In addition, viral interference was documented *in vitro* with co-infection of herpes simplex virus (51), bovine herpesvirus (36)(37), and equine herpesvirus (13). However, there is no detailed study on the influence of recombination or viral interference on vaccine efficacy.

The Rispens strain, also called CVI988, belongs to MDV1 and has been used as a vaccine against MD as well as HVT. Mixtures of Rispens with HVT have been used for protection against highly virulent MDV strains for over 20 years without any reports of recombination (5)(65). Furthermore, the Rispens strain was reported to have no negative effect on the protective efficacy against IBDV and MDV challenge when inoculated into chickens simultaneously with recombinant HVT/IBD (29). Based on these observations, recombinant Rispens strains were developed in our laboratory as new vectors that can be used simultaneously with HVT. Recombinant Rispens strains carrying the *VP2* gene of IBDV (Rispens/IBD) driven by five different promoters were constructed, and the efficacy of the viruses against IBDV challenge has been evaluated (Chapter 1). Among these five viruses, Rispens/IBD driven by the Coa5 promoter, which is derived from chicken beta-actin promoter, provided the highest protection against IBDV challenge (Chapter 1).

To evaluate the potential of the multivalent vaccination approach using my recombinant Rispens viruses, the compatibility between Rispens/IBD and HVT/ND was evaluated in this study. I hypothesized that simultaneous vaccination with Rispens/IBD and HVT/ND would not cause interference on protective efficacy. Rispens/IBD was inoculated into chickens simultaneously with HVT/ND, and the efficacy of this combination was evaluated against IBDV, NDV, and MDV challenge. Furthermore, the potential for recombination and interference of these two viruses in the inoculated chickens was investigated.

MATERIALS AND METHODS

Viruses and cells. The parental Rispens strain was obtained from the Central Veterinary Institute of Wageningen, the Netherlands (46). Rispens/IBD harbors the IBDV *VP2* gene driven by the *Coa5* promoter in its *US2* region, and was previously constructed in our laboratory (Chapter 1). HVT/ND is a recombinant HVT expressing the F protein of NDV, and was previously constructed in our laboratory (14). The parental Rispens strain, and the recombinant Rispens/IBD and HVT/ND viruses were propagated in chicken embryo fibroblasts (CEFs) prepared from 10-day-old specific-pathogen-free (SPF) chicken embryos (Nissei Bio Company, Yamanashi, Japan). CEFs were maintained in a 1:1 mixture of Leibovitz's L-15 (Life Technologies Corporation, Tokyo, Japan) and McCoy's 5A Medium (Life Technologies Corporation) supplemented with 4% calf serum. The IBDV standard challenge (STC) strain used for IBDV challenge, and the NDV Texas GB strain used for NDV challenge were obtained from the National Veterinary Services Laboratories, U.S. Department of Agriculture/Animal and Plant Health Inspection Service (Ames, IA, USA). The highly virulent RB-1B strain of MDV was obtained from Dr. K. A. Schat, Cornell University, Ithaca, NY, and used for the MDV challenge. The use of recombinant virus was confirmed by Ministry of Education, Culture, Sports, Science and Technology (H.24 #2199).

Efficacy trial 1. To assess the efficacy of simultaneous vaccination with Rispens/IBD and HVT/ND against IBDV or NDV challenge (efficacy trial 1), 1-day-old commercial layer chickens (KANAGAWA YOUKEIREN, Kanagawa, Japan) with maternally derived immunity against IBDV and NDV were divided into five groups. Chicks in Group 1 (non-immunized, non-challenged negative control) and Group 2 (non-immunized, challenged positive control) were left unvaccinated. Chicks in Groups 3 to 5 were inoculated subcutaneously with one dose of Rispens/IBD alone, a mixture of Rispens/IBD and HVT/ND, and HVT/ND alone, respectively. The blood was collected from all chickens once a week between the ages of 1 day and 7 weeks, and all sera were tested for the presence of anti-IBDV antibodies with a commercial IBDV enzyme-linked immunosorbent assay (ELISA) kit (FlockChek IBD; Idexx Laboratories, Tokyo, Japan) and for the presence of anti-NDV antibodies with a commercial NDV ELISA kit (ID SCREEN NEWCASTLE DISEASE INDIRECT; IDVet, Grabels, France). At 7 weeks post-vaccination, challenge with virulent IBDV or virulent NDV was conducted. All chickens in Group 3 and half of the chickens in Groups 2 and 4 were challenged orally with 10^3 of the 50% egg infectious dose (EID₅₀) of the virulent STC IBDV strain, whereas all chickens in Group 5 and the remaining chickens in Groups 2 and 4 were challenged with 10^3 of the EID₅₀ of the highly virulent Texas GB NDV strain via intramuscular

injection into the femoral region. Following IBDV challenge, the chickens were checked daily for clinical signs associated with IBD. Seven days post-IBDV challenge, the challenged chickens and half of the Group 1 (non-immunized, non-challenged negative control) chickens were euthanized and necropsied for observations of grossly observable bursal lesions such as edema, discoloration, atrophy, hemorrhage, and yellow or gelatinous exudates. The body and bursa weights were measured in order to calculate the B/B index, which is the ratio between the weight of the bursa and the body weight of challenged chickens divided by the same ratio for non-challenged chickens. Following NDV challenge, the chickens were observed daily for clinical signs typical of neurotropic velogenic ND, such as depression, gasping, neurological symptoms, moribund, and mortality, for 14 days.

Efficacy trial 2. To assess the efficacy of simultaneous vaccination with Rispons/IBD and HVT/ND against MDV challenge (efficacy trial 2), 1-day-old SPF chickens (Nissei Bio Company) were divided into two groups. Chicks in Group 1 were inoculated subcutaneously with one dose of the mixture of Rispons/IBD and HVT/ND, and the chicks in Group 2 (non-immunized, challenged positive control) were left unvaccinated. At 4 days post vaccination, all chickens were challenged subcutaneously with 3×10^3

plaque-forming units of the highly virulent MDV strain RB-1B. The chickens were checked daily for clinical signs associated with MDV for 6 weeks. At 7 weeks of age, the chickens were euthanized and necropsied to monitor grossly observable lesions and tumors associated with MD in various tissues, including the spleen, kidneys, liver, heart, or gonad.

Virus isolation from blood lymphocytes. Virus isolation was performed using blood lymphocytes collected from three chickens vaccinated with the mixture of Rispens/IBD and HVT/ND between 3 and 7 weeks after vaccination in efficacy trial 1. The blood lymphocytes were inoculated into a CEF monolayer and cocultured until plaques were observed. The plaques were then assessed for expression of the IBDV VP2 protein and the NDV F protein by an immunofluorescence assay.

Immunofluorescence assay. Plaques on the CEF monolayer were fixed with a methanol:acetone mixture (1:2). The cells were incubated for 1 h at 37°C with a mixture of mouse anti-IBDV VP2 monoclonal antibody R63 (54) and rabbit anti-NDV F serum (14), followed by incubation with a mixture of Alexa Fluor 546 anti-mouse IgG (Invitrogen, Tokyo, Japan) and Alexa Fluor 488 anti-rabbit IgG (Invitrogen); all

antibodies were used at a 500-times dilution. Plaques were then identified for expression of IBDV VP2 or NDV F protein by fluorescence microscopy (Olympus IX70 Inverted Fluorescence Microscope; Olympus Corporation, Tokyo, Japan).

Quantitative estimation of virus load with quantitative polymerase chain reaction (qPCR). The compatibility between Rispons/IBD and HVT/ND was also analyzed by qPCR. In efficacy trial 1, blood lymphocytes were collected from three chickens in Group 2 (non-immunized) and from five chickens each from Groups 3 through 5 at 3 and 5 weeks of age. DNA was extracted from 1×10^7 cells of the blood lymphocytes using QiaAmp DNA Mini kit (QIAGEN, Tokyo, Japan). The extracted DNA was analyzed via triplex qPCR using the Taqman system targeting chicken ovotransferrin, HVT SORF1, and MDV-1 Meq. The primer and probe sets used in qPCR are shown in Table 2-1. Differences between the groups were evaluated by the Steel-Dwass test using R software (version 3.2.2).

Ethical statement. Prior to carrying out the animal experiments, the study designs and procedures were approved by the ethics committee of Ceva Animal Health. All animal procedures strictly followed the company's guidelines for research with animals.

RESULTS

Efficacy against IBDV or NDV challenge. A covaccination experiment was performed to assess compatibility between Rispons/IBD and HVT/ND. Challenge was conducted in chickens at 7 weeks of age with either virulent IBDV or virulent NDV (efficacy trial 1). In the IBDV challenge, all the chickens in the non-immunized, challenged positive control (Group 2) developed gross bursal lesions such as atrophy, whereas no chickens in the covaccinated group (Group 4) developed lesions, resulting in 100% protection (17/17), which was equivalent to the protection conferred by Rispons/IBD alone (Group 3) (Table 2-2). Covaccination also provided good protection in the NDV challenge (Table 2-2). Both the covaccinated group (Group 4) and the HVT/ND-alone vaccinated group (Group 5) showed 94% (16/17) protection against NDV challenge, although one bird in each group died from NDV. By contrast, all chickens in the challenged positive control group (Group 2) showed clinical signs of ND and 13/15 chickens died. Anti-IBDV and anti-NDV antibody titers increased in the covaccinated group in a similar manner as those in the Rispons/IBD- or HVT/ND-alone groups after maternal antibodies declined through 3 weeks of age (Figs. 2-1 and 2-2). These results indicated a lack of interference on protective efficacy between Rispons/IBD and HVT/ND.

Efficacy against MDV challenge. In the MDV challenge (efficacy trial 2), one out of 18 birds from the covaccination group (Group 1) died, whereas 14 out of 18 birds from the non-immunized, challenged positive control group (Group 2) died. In Group 2, all of the other surviving birds showed gross lesions, while none of the surviving birds in Group 1 showed any gross lesions. As a result, covaccination (Group 1) provided 94 % protection, whereas no bird in Group 2 was protected. This result confirmed that covaccination of Rispens/IBD and HVT/ND was also effective for MDV challenge.

Virus isolation from blood lymphocytes. The stability of Rispens/IBD and HVT/ND was assessed by an immunofluorescence assay using the blood lymphocytes collected from chickens covaccinated with Rispens/IBD and HVT/ND. Plaques expressing IBDV VP2 were observed in all of the chickens examined, whereas plaques expressing NDV F were observed in all but one chicken (#3) at 3 and 4 weeks of age (Table 2-3). No plaques expressing both IBDV VP2 and NDV F were found; i.e., the all plaques stained either for IBDV VP2 or for NDV F protein and no plaques were unstained. However, the ratio of the stained plaques fluctuated. In chicken #1, the approximate ratios of the plaques stained for F:VP2 were 2:8 (3 weeks of age), 5:5 (4, 5, and 7 weeks of age), and 8:2 (6 weeks of age). In chicken #2, these ratios were 8:2 (3 and 4 weeks of age), 7:3 (5 and 6 weeks of

age), and 9.5:0.5 (7 weeks of age). In chicken #3, they were 0:10 (3 and 4 weeks of age), 9:1 (5 weeks of age), 8:2 (6 weeks of age), and 6:4 (7 weeks of age).

Virus quantitation from blood lymphocytes. Compatibility between Rispens/IBD and HVT/ND was assessed by qPCR using the blood lymphocytes collected from chickens covaccinated with Rispens/IBD and HVT/ND. No statistically significant difference was observed with respect to HVT copy numbers between the HVT/ND-alone group and the covaccinated group at 3 and 5 weeks of age (Figs. 2-3A and 3B), although only small amounts of HVT DNA were detected. Similarly, Rispens copy numbers of the covaccinated group were comparable to those of the Rispens/IBD-alone group at 3 and 5 weeks of age (Figs. 2-3C and 3D). These results suggested that the presence of HVT/ND did not interfere with replication of Rispens/IBD in chickens, and vice versa.

DISCUSSION

In this chapter, the mixed vaccination of Rispens/IBD and HVT/ND showed excellent protection against IBDV, NDV, and MDV challenge. Furthermore, no interference in the covaccinated chickens was revealed by the qPCR with the blood lymphocytes collected from the chickens. In addition, all plaques formed by the blood lymphocytes expressed either VP2 or F protein, suggesting that Rispens/IBD and HVT/ND were stable in chickens and that no recombination occurred in the covaccinated chickens. A previous study showed that mixture of an HVT vector vaccine and Rispens induced an immune response against MDV and the inserted antigen in HVT (29). Similarly, the present study demonstrated that the mixture of a Rispens vector vaccine and an HVT vector vaccine could induce an immune response against both of the inserted antigens and MDV.

Recombination of two related viruses has been reported in *Alphaherpesvirinae*. In herpes simplex virus and bovine herpesviruses, two distinct virus serotypes could recombine when inoculated into animals or cultured cells simultaneously (36)(42). In the present study, viral plaques isolated from chickens vaccinated with the mixture of Rispens/IBD and HVT/ND expressed strictly either one of the other antigens, NDV F protein or IBDV VP2 protein, and no plaques expressed both. This result indicated that recombination of HVT/ND and Rispens/IBD, which both belong to the *Mardivirus* genus,

did not occur in the covaccinated chickens. There are two possible explanations for this result. First, recombination of distinct viruses would require coinfection of the viruses in a single cell. However, inhibition of viral superinfection may have occurred at the cellular level in the vaccinated chickens, so that the two distinct viruses would have had little opportunity to infect the same cells. A previous study showed that most of the feather follicle epithelial cells of chickens inoculated simultaneously with two RB-1B strains were infected with only one or the other virus and coinfection of the viruses was observed in only a portion of cells (21). Second, the homology between the genomes of HVT and Rispens is not very high because they belong to the same genus but are two distinct viruses. Therefore, the recombination rate would be expected to be low, even if one cell could accommodate both viruses. A previous study that focused on interspecific recombination revealed that no recombination occurred between ruminant alphaherpesviruses and bovine herpesviruses and a high degree of genetic relatedness was required for successful interspecific recombination (36). Taken together, these findings suggest that the mixture of HVT/ND and Rispens/IBD is a safe vaccine without major risk of recombination.

Following virus isolation from the blood lymphocytes, I did not detect plaques expressing NDV F protein from one chicken (#3) at 3 and 4 weeks of age. However, this

lack of detection is not considered to reflect interference between HVT/ND and Rispons/IBD. Instead, this was likely due to fluctuations in the ratio of the plaques expressing either NDV F protein or IBDV VP2 protein, which also showed variation over the experimental period in chickens #1 and #2, and there was no dominant overgrowth of either virus. This result suggested that viral distribution and growth intrinsically fluctuate depending on individual and age, and that the viral number may be below the detection limit in a given period, despite continuous infection with the virus. A similar phenomenon was observed with a single inoculation of HVT in chickens in previous work. Handberg *et al.* reported that HVT could not be detected from the blood lymphocytes of some chickens at some ages, even though these chickens were actually infected with HVT (19). Therefore, the lack of detection of F-expressing plaques in chicken #3 was not considered to be caused by interference with Rispons/IBD but was instead considered an intrinsic characteristic of HVT.

The results of qPCR showed that the detected viral copy number of HVT/ND was lower than that of Rispons/IBD. However, excellent protection was observed against the NDV challenge in both the single-vaccinated group and the covaccination group, suggesting that the chickens were infected with an adequate amount of HVT/ND to induce an immune response against NDV. PCR is considered to be a more sensitive method for

detecting Rispens compared to cultivation, although the opposite trend has been observed with respect to HVT (19). Together with the results from the viral isolation experiment, it is considered that the differences in viral copy numbers between HVT/ND and Rispens/IBD were not caused by a difference in the infection rate of the circulating lymphocytes, but rather by the difference in the sensitivity of PCR analysis for these viruses.

The IBDV ELISA titers and NDV ELISA titers of single vaccinations showed similar curves as that of the mixed vaccination, suggesting no interference of the mixed vaccination. However, the IBDV ELISA titers of chickens inoculated with Rispens/IBD or the mixture of Rispens/IBD and HVT/ND at 2 and 3 weeks of age were lower than the cutoff value of 0.2. Nevertheless, this does not necessarily indicate that these vaccinations are ineffective at an earlier stage than 7 weeks of age; in Chapter 1, a Rispens/Bac-IBD showed an excellent protection level at 5 weeks of age in spite of the lower IBDV ELISA titer than the cutoff value measured by the same ELISA kit used in the present study. In addition, I compared the IBDV ELISA titers at 7 weeks of age using another ELISA kit (ID SCREEN IBD VP2; IDVet) with those obtained from the kit used in the present study. As a result, titers of the ID SCREEN IBD VP2 ELISA kit were about three times higher than those of the kit used in this study, even though the cutoff values of both kits are the

same (data not shown). In the IDVet ELISA kit, the bottoms of the wells were covered with VP2 protein but those of the ELISA kit used in this experiment were coated with whole particles of IBDV. However, further experiments will be needed to clarify these results, such as a challenge at 5 weeks with mixed inoculation.

In summary, I discovered that mixed inoculation of HVT/ND and Rispens/IBD is a useful tool to induce immunity against MDV, IBDV, and NDV. This mixed inoculation with recombinant Rispens and recombinant HVT provides a new vaccination strategy for development of an effective polyvalent vaccination program.

Table 2-1. Sequences of the primers or the probes used for quantitative PCR.

Primer or probe name	Sequence (5'-3')
Ovo 1F	CACTGCCACTGGGCTCTGT
Ovo 1R	GCAATGGCAATAAACCTCCAA
ChOvo-Probe2	AGTCTGGAGAAGTCTGTGCAGCCTCCA
HVT-Sorf1 F5	AAACGTACGTCCAAGCAAGC
HVT-Sorf1 R5	ACGTTCCCTCGTTCAGGTT
Sorf1-Probe	TACGATCACGTACAGTCCCGCGT
Meq 1F	GGTCTGGTGGTTTCCAGGTG
Meq 1R	GCATAGACGATGTGCTGCTGA
Meq-Probe2	AGACCCTGATGATCCGCATTGCGACT

Table 2-2. Protective effect of Rispens/IBD and HVT/ND against virulent IBDV or NDV challenge.

Group number	Virus used for vaccination	Number of chickens	IBDV challenge				NDV challenge		
			B/B Index ^a (SD)	Number of chickens with bursal lesions/total number	Number of dead chickens after challenge	% protection	Number of chickens with ND clinical signs/total number	Number of dead chickens after challenge	% protection
1	NINC ^b	15	1.00 (0.26)	N/A ^c	N/A	N/A	N/A	N/A	N/A
2	NICC ^d	30	0.60 (0.23)	15/15	8/15	0%	15/15	13/15	0%
3	Rispens/IBD	17	1.13 (0.28)	0/17	0/17	100%	N/A	N/A	N/A
4	Rispens/IBD and HVT/ND	34	1.25 (0.27)	0/17	0/17	100%	1/17	1/17	94%
5	HVT/ND	17	N/A	N/A	N/A	N/A	1/17	1/17	94%

^aB/B Index = the bursa weight to body weight ratio (BB ratio) of infected birds/the BB ratio of NINC;

^bNINC = non-immunized, non-challenged negative controls; ^cN/A = not analyzed; ^dNICC = non-immunized, challenged positive controls

Table 2-3. Presence or absence of plaques expressing NDV F protein or IBDV VP2 protein formed by viruses isolated from co-vaccinated chickens.

Chicken No.	Age of chickens									
	3 weeks		4 weeks		5 weeks		6 weeks		7 weeks	
	F	VP2	F	VP2	F	VP2	F	VP2	F	VP2
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+
3	-	+	-	+	+	+	+	+	+	+

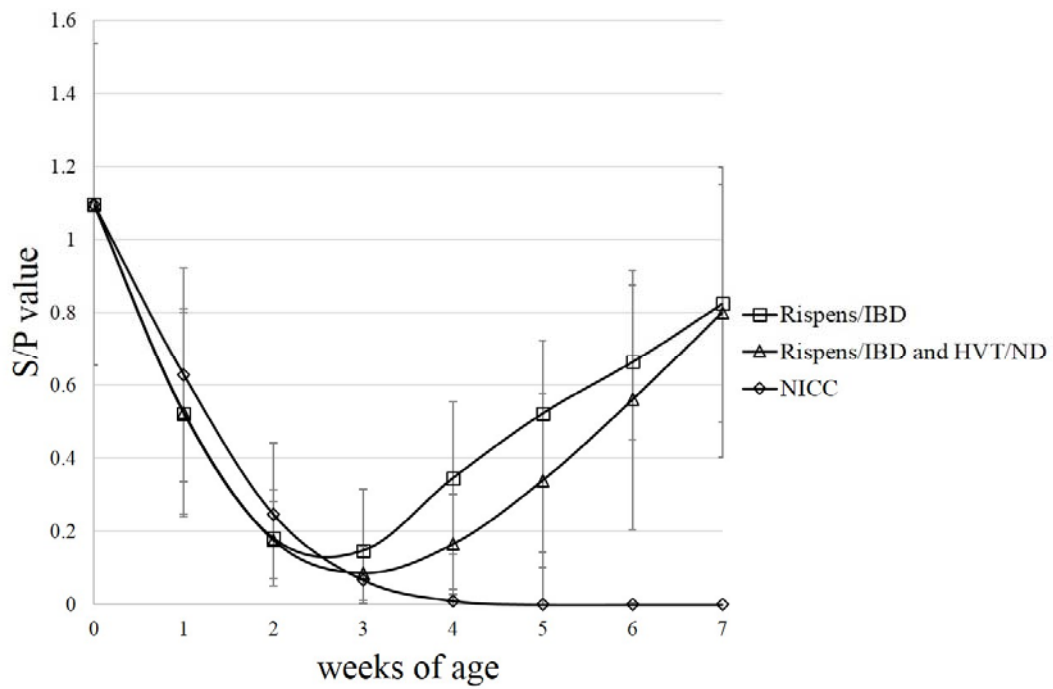


Figure 2-1. IBDV titers in chickens vaccinated with Rispens/IBD or a mixture of Rispens/IBD and HVT/ND using a commercial IBD enzyme-linked immunosorbent assay kit (Idexx Laboratories, FlockChek IBD).

Sample-to-positive (S/P) values were calculated according to the manufacturer's protocol. The cutoff value of seroconversion was 0.2. NICC = non-immunized, challenged positive control.

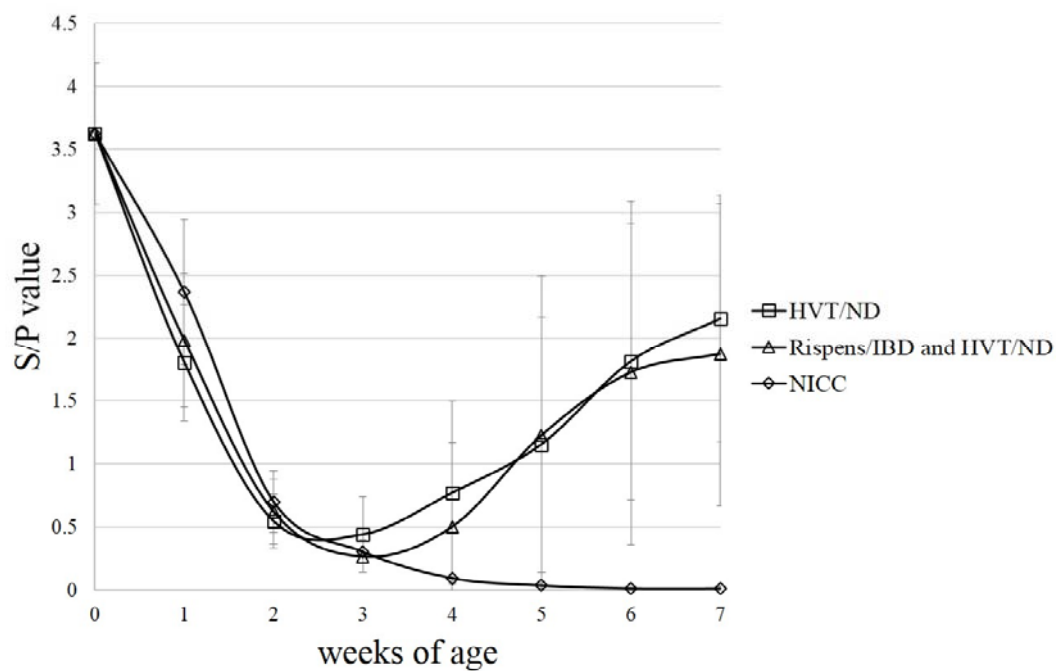


Figure 2-2. NDV titers in chicks vaccinated with HVT/ND or a mixture of Rispens/IBD and HVT/ND using a commercial ND enzyme-linked immunoassay kit (ID SCREEN NEWCASTLE DISEASE INDIRECT, IDVet).

The serum samples were used at a dilution of 1:3 before analysis. Sample-to-positive (S/P) values were calculated according to the manufacturer's protocol. NICC = non-immunized, challenged positive control.

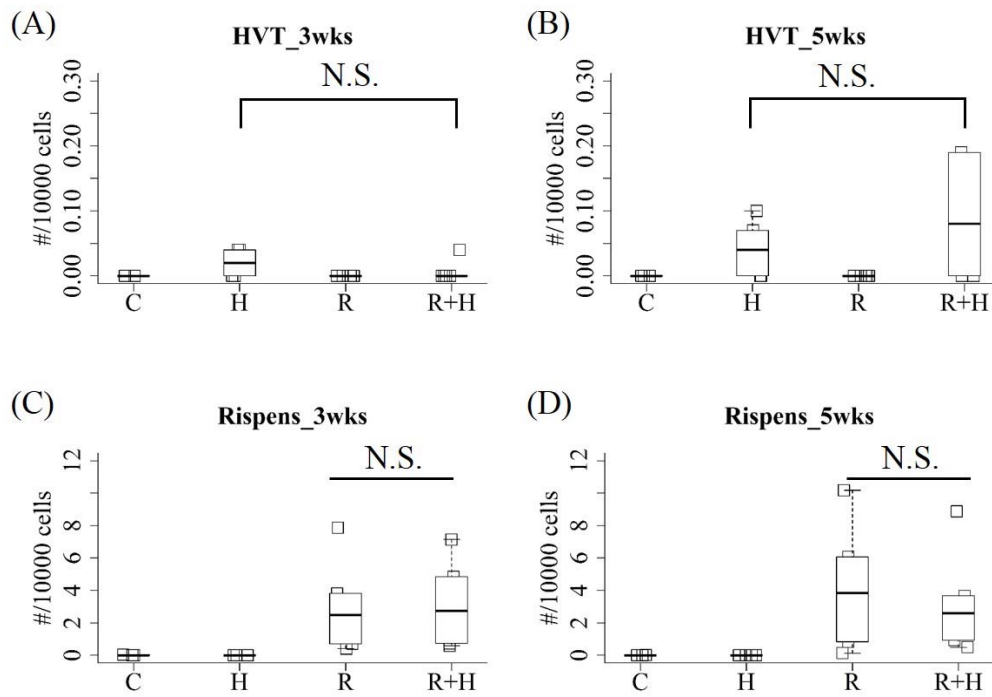


Figure 2-3. The viral copy numbers in the peripheral blood. The copy numbers of HVT and Rispens viruses were evaluated using qPCR at 3 and 5 weeks of age.

(A) The copy numbers of HVT in 3 weeks-old chicks. (B) The copy numbers of HVT in 5 weeks-old chicks. (C) The copy numbers of Rispens in 3 weeks-old chicks. (D) The copy numbers of Rispens in 5 weeks-old chicks. C: Non-immunized, non-challenged negative controls (NINC). H: Group inoculated with HVT/ND. R: Group inoculated with Rispens/IBD. R+H: Group inoculated with Rispens/IBD and HVT/ND. N.S.: not significant by the Steel-Dwass test.

Chapter 3

**Removal of Inserted BAC after linearization (RIBON) – a novel strategy to
excise the mini-F sequences from viral BAC vectors**

ABSTRACT

The bacterial artificial chromosome (BAC) technology has been a mainstay approach for generating recombinant viruses, and several methods for excision of the mini-F sequences from the viral BAC vectors have been developed. However, these strategies either require complicated procedures or leave scars of inserted sequences. To overcome these problems, a new method to excise the mini-F sequences from viral BAC vectors based on the Removal of Inserted BAC after linearizatiON (RIBON) strategy was developed in this chapter for herpesvirus of turkeys (HVT). Enhanced green fluorescent protein (eGFP) DNA and the mini-F sequences were inserted into the gene encoding HVT thymidine kinase (TK) by homologous recombination in chicken embryo fibroblasts (CEFs), and the constructed HVT-BAC vector was used to transform *Escherichia coli* (pHVT-BAC). To remove the inserted eGFP and mini-F sequences, pHVT-BAC was linearized using a homing endonuclease I-*SceI* and used to cotransfect CEFs together with a plasmid containing the TK gene of HVT. The obtained viruses (44%) did not express eGFP, and DNA sequencing of isolated clones revealed that they were completely free of the inserted BAC sequences. Moreover, growth kinetics and plaque morphology of reconstituted viruses were comparable with those of the parental HVT. The results of this chapter demonstrate

that the novel RIBON approach to remove mini-F sequences from the viral genome is simple and effective.

INTRODUCTION

Herpesviruses have frequently been used for generating recombinant vaccines because of their capacity to accommodate foreign genes and maintain persistent infection in the host. In the past, recombinant herpesviruses had been constructed by laborious homologous recombination in eukaryotic cells; however, the adaptation of the bacterial artificial chromosome (BAC) technology for viruses in 1993 (31) provided the mainstay approach to generate recombinant viruses.

The use of the BAC technology for virus cloning requires the excision of the minimal fertility factor replicon (mini-F), the backbone of the BAC vector, from the viral genome-containing construct. To achieve this, four methods have been mainly used (56). The most popular one utilizes the *Cre/loxP* or *FLP/FRT* recombination systems (1)(6). In these systems, *loxP* or *FRT* sites are added to either end of the mini-F sequences; then, one of each of the *loxP* or *FRT* sites and sandwiched mini-F sequences are removed by Cre or FLP enzymes via recombination. For this reaction, the virus BAC should be either incubated with Cre or FLP enzymes *in vitro* or transferred into eukaryotic cells together with the Cre or FLP expression plasmids. Although the method is easy, it introduces one 34-bp *loxP* or *FRT* sequence, which can compromise the development of commercial vaccines and may change the

expression of viral genes if inserted into open reading frames or gene regulatory regions. In addition, some reconstituted viruses retain the mini-F sequences; then, selective purification of mini-F-negative viruses is required, because the FLP and Cre reactions tend to approach an equilibrium, resulting in the same emergence rate for mini-F- negative and -positive viruses. The second method uses the recombination mechanism of eukaryotic cells and a repair vector or PCR product substitute for the mini-F sequences (47). This method requires the repair vector or PCR product homologous to the original sequence upstream and downstream of the insertion site for the mini-F sequences. The repair vector or PCR product and the virus BAC are cotransferred into eukaryotic cells, where the mini-F sequences are removed via recombination between homologous sequences of the repair vector or PCR product and the virus BAC. To obtain a homogeneous mini-F-negative viral population, laborious purification steps are required, but no residual mini-F sequences are left at the insertion site. The third and fourth methods use the recombination mechanism of eukaryotic cells and the sequence overlapping the mini-F replicon; these methods do not require laborious purification steps and leave no scar. In the third method, the mini-F is sandwiched between homologous sequences (59), which recombine with each other and remove the mini-F during virus replication in eukaryotic cells. The

drawback of this strategy is the instability of virus BAC in *Escherichia coli* due to duplication of the viral sequence. To overcome this problem, the fourth method utilizes two sets of inverted duplicated sequences (9), providing stable maintenance of the mini-F in *E. coli*.

Although the fourth method seems to be an ideal strategy, the construction of the virus BAC is complicated by inverted sequence duplications, making this strategy difficult to use once the virus BAC is constructed by other methods. In this chapter, I developed a simple method that did not leave mini-F scars and could be used after virus BAC construction; it is called the Removal of Inserted BAC after linearizatiON (RIBON) and is based on the excision of the mini-F cassette using the recombination mechanism of eukaryotic cells, a repair vector, and linearized virus BAC DNA.

MATERIALS AND METHODS

Chicken embryo cells and herpesvirus. Chicken embryo fibroblasts (CEFs) were obtained from specific pathogen-free (SPF) 10-day-old chicken embryos (Nissei Bio Company, Yamanashi, Japan) and maintained in a 1:1 mixture of Leibovitz's L-15 and McCoy's 5A Medium (Life Technologies Corp., Tokyo, Japan) (LM) supplemented with 4% calf serum (CS). The parental herpesvirus of turkeys (HVT) FC126 strain (66) was obtained from Dr R. L. Witter at the Avian Disease and Oncology Laboratory (East Lansing, MI, USA). The use of recombinant virus was confirmed by Ministry of Education, Culture, Sports, Science and Technology (H.27 #221).

Construction of pUC18-HVT-TK. A 3.3-kb DNA fragment of the HVT FC126 genome comprising the region from the UL22 to UL25 gene (nucleotides [nts] 45,700 to 48,967 of the FC126 genome, GenBank Accession # AF291866) was cloned by PCR using primers F-HVT-*SalI*-45700 and R-HVT-*SacI*-48967 (Table 3-1) and FC126 DNA as a template. The amplified fragment was digested with *SalI* and *SacI* and cloned into the pUC18 vector (TaKaRa Bio, Shiga, Japan), resulting in pUC18-HVT-TK (Fig. 3-1A).

Construction of pUC18-HVT-TK-*Sfi*I. The pUC18-HVT-TK-*Sfi*I plasmid also comprises the UL22-UL25 region, with the *Sfi*I recognition site added between nts 47,316 and 47,317. Two primer pairs, F-HVT-*Sal*I-45700/R-HVT-*Sfi*I-47316 and F-HVT-*Sfi*I-47317/R-HVT-*Sac*I-48967 (Table 3-1), were used for amplification of the UL22–UL25 region with the *Sfi*I site. The resultant 3.3-kbp fragment was cloned into the pUC18 vector (TakaRa Bio) digested with *Sal*I and *Sac*I, resulting in pUC18-HVT-TK-*Sfi*I (Fig. 3-1B).

Construction of pUC18-HVT-BAC. The CMV promoter of pBK-CMV (Stratagene, Tokyo, Japan) with the added *Sfi*I restriction site and the enhanced green fluorescent protein (eGFP) gene of pEGFP-1 (TaKaRa Bio) with added *Sal*I and *Sfi*I restriction sites were amplified by PCR using two primer pairs (F-*Sfi*I-*LoxP*-CMV/R-eGFP-CMV and F-CMV-eGFP/R-*Sfi*I-*LoxP*-*Sal*I-eGFP, respectively, Table 3-1). The products were mixed and used as a template for the following PCR with primers F-*Sfi*I-*LoxP*-CMV/R-*Sfi*I-*LoxP*-*Sal*I-eGFP, producing the CMV-eGFP fragment. As a result, the *Sfi*I recognition site and *loxP* sequence were added to the 5' end, while the *Sal*I site, another *loxP* sequence, and *Sfi*I site were added to the 3' end of the fragment, which was then cloned into the T-cloning site of the T-vector pMD19 (TaKaRa Bio)

to generate pCMVeGFP. The mini-F and chloramphenicol resistance cassette obtained from pBeloBAC11 (New England BioLabs, Tokyo, Japan) by *SalI* digestion were cloned into the *SalI* restriction site of pCMVeGFP, resulting in pCMVeGFP-BAC.

To insert the *I-SceI* recognition site and 50-bp duplication sequence (nts 47,317–47,366 of FC126) adjacent to the *SfiI* recognition site of pUC18-HVT-TK-*SfiI* (Fig. 3-1B), a 100-bp DNA fragment was amplified without a template using complimentary primers, F-TK-duplication and R-TK-*SfiI*-*I-SceI* (Table 3-1). The amplified fragment was cloned into the *NaeI* site of pUC18-HVT-TK-*SfiI* using the In-Fusion HD Cloning Kit (TaKaRa Bio), resulting in the pUC18-HVT-TK-*I-SceI*-*SfiI* plasmid (Fig. 3-1C), which contained the 45,700–48,967 fragment with the 50-bp duplication sequence, *I-SceI* site, and *SfiI* site inserted between nts 47,316 and 47,317.

Then, CMV promoter, eGFP, mini-F, and chloramphenicol resistance sequences obtained from pCMVeGFP-BAC by *SfiI* digestion were cloned into the *SfiI* site of pUC18-HVT-TK-*I-SceI*-*SfiI*, resulting in pUC18-HVT-BAC (Fig. 3-1D).

Construction of HVT-BAC. CEFs (1×10^7) were cotransfected with 1 μ g of

pUC18-HVT-BAC and 2 µg of the FC126 genomic DNA by electroporation using Nucleofector II (Lonza, Basel, Switzerland), diluted in 20 mL LM, and seeded in two 96-well tissue culture plates. Five days post-transfection, eGFP-positive plaques were identified, and the cells were detached by trypsinization, mixed with fresh CEFs in 10 ml LM, and seeded in a 96-well plate. After three rounds of purification by limited dilution, recombinant virus clones (HVT-BAC) were isolated, and HVT-BAC DNA was extracted from CEFs infected with the purified viruses as previously described (39) and used to transform *E. coli* GS1783 strain (57) (obtained from Dr G. Smith, Northwestern University, Chicago, IL, USA) by electroporation at 1.6 kV, 25 µF, and 200 ohm using Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA). The transformed bacteria were grown with agitation for 1 hr at 30°C, planted on Luria-Bertani (LB) agar containing 20 µg/mL chloramphenicol, and incubated overnight at 30°C. The emerged colonies were inoculated into liquid LB medium, and circular HVT-BAC DNA (pHVT-BAC; Fig. 3-2A) was purified by the alkaline lysis method and analyzed by band pattern after digestion with *Eco*RI and separation on 0.8% agarose gels.

Reconstitution of HVT and HVT-*Sfi*I. pHVT-BAC DNA extracted from 3 ml of

overnight culture of GS1783 cells was digested with a homing endonuclease I-*SceI* (New England Biolabs) and used together with 1 µg of pUC18-HVT-TK-*SfiI* or pUC18-HVT-TK to cotransfect CEFs by electroporation. The transfected cells (1×10^7) were diluted with 20 mL LM and seeded into two 96-well plates. Five days after transfection, plaques with or without eGFP expression were identified.

Identification of BAC excision. Reconstituted (rc) HVT-*SfiI* viruses (rcHVT-*SfiI*) rescued by cotransfection of linearized pHVT-BAC and pUC18-HVT-TK-*SfiI* were passaged in CEFs 10 times and examined for eGFP expression. DNA was extracted from CEFs infected with rcHVT-*SfiI* at the second passage using the QIAamp DNA Mini Kit (Qiagen, Limburg, The Netherlands), and subjected to further analysis. Extracted DNA carrying or not carrying eGFP, mini-F, and chloramphenicol resistance sequences were identified by PCR; three primer pairs (F-eGFP/R-BAC, F-cm/R-HVT-47750, and F-HVT-46898/R-HVT-*SacI*-48967; Table 3-1) were used to amplify the regions between the eGFP-encoding gene and mini-F sequences, the chloramphenicol resistance cassette and insertion site of the FC126 genome, and the insertion site region of the FC126 genome, respectively. FC126 genomic DNA was also used as a template for a positive control. The products amplified with primers F-

eGFP/R-BAC and F-cm/R-HVT-47750 were directly separated on 0.8% agarose gels, while the fragment amplified with primers F-HVT-46898/R-HVT-*SacI*-48967 was first digested with *SfiI* before separation in the same gel. The *SfiI*-undigested fragments were excised, DNAs were extracted using the NucleoSpin Gel and PCR Clean-up kit (TaKaRa Bio), and were sequenced using F-HVT-46898 or R-HVT-*SacI*-48967 primers and a CEQ 2000XL sequencer (Beckman Coulter, Tokyo, Japan).

Isolation of rcHVT. To purify the cloned virus, CEFs infected with rcHVT rescued by cotransfection with pHVT-BAC and pUC18-HVT-TK were trypsinized and sonicated in sucrose, phosphate, glutamate, and albumin (SPGA) buffer (4). Cell-free viruses in SPGA were inoculated into fresh CEFs in 96-well plates and incubated for 2 hr at 37°C; then, the supernatant was removed, and LM supplemented with 4% CS was added to CEF monolayers. Five days after infection, cells with or without plaques and eGFP expression were identified; DNA was extracted and analyzed by PCR using primer pairs F-eGFP/R-BAC, F-cm/R-HVT-47750, and F-HVT-46898/R-HVT-*SacI*-48967 (Table 3-1) described above.

Growth kinetics. CEFs plated in 6-well plates (9.5×10^5 cells/well) were infected

with rcHVT or FC126 at the multiplicity of infection (MOI) 0.001. At 0, 24, 48, 72, and 96 hr after infection, CEFs were harvested, and virus titers were determined by the plaque assay. The data were obtained from two independent experiments. Viral growth kinetics was evaluated by Student's *t*-test.

Plaque assay. CEFs seeded in 12-well plates (1×10^6 cells/well) were infected with serial 10-fold dilutions of virus-carrying trypsinized cells and grown in LM supplemented with 4% CS for 4 days. Cells were then fixed with methanol:acetone mixture (1:2) and incubated with anti-FC126 monoclonal antibody L78 diluted 1:500 (28) for 1 hr at 37°C; biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody. The signal was detected using the VECTASTAIN ABC-AP kit (Vector Laboratories) for signal enhancement and NBT/BCIP solution (Roche Applied Science, Penzberg, Germany) for development. Plaque numbers were determined macroscopically, and plaque morphology was evaluated. The average size of 50 plaques was calculated using the CellSens standard program (Olympus, Tokyo, Japan). Plaque sizes were compared using Student's *t*-test.

RESULTS

Construction of HVT-BAC. After insertion of eGFP, chloramphenicol resistance cassette, and mini-F sequences into the TK region of FC126 by homologous recombination in CEFs, four independent clones of HVT-BAC were isolated, and the extracted viral DNAs were used to transduce *E. coli* GS1783 producing a total of 25 colonies. The analysis of DNA extracted from *E. coli* (pHVT-BAC; Fig. 3-2A) revealed two band patterns shared by clones #1, #2, and #4, and clones #3 and #5, respectively (Fig. 3-2B), although all band patterns were similar to that of pHVT-BAC (Fig. 3-2C). Therefore, clones pHVT-BAC#1 and pHVT-BAC#3 were selected for further analysis as representatives of the two band patterns.

Reconstitution of HVT-*Sfi*I and identification of BAC excision. To excise eGFP, chloramphenicol resistance cassette, and the mini-F, pHVT-BAC DNA was first digested with the homing enzyme I-*Sce*I, which has a single recognition site of 18-bp in pHVT-BAC adjacent to the 50-bp duplication site (Fig. 3-2A). Linearized pHVT-BAC #1 or #3 and pUC18-HVT-TK-*Sfi*I were used to cotransfect CEFs. In this study, pUC18-HVT-TK-*Sfi*I carrying the *Sfi*I site in the TK region was used to distinguish the viruses recombined with this plasmid from those reconstituted by self-

recombination of pHVT-BAC via the 50-bp duplication sequence.

Five days after transfection, one plaque was produced by pHVT-BAC#1 (rcHVT-*SfiI*#1a) and eight plaques—by pHVT-BAC#3 (rcHVT-*SfiI*#3a, 3b, 3c, 3d, 3e, 3f, 3g, and 3h). Four of the nine viruses were not fluorescent (44%, rcHVT-*SfiI*#3a, 3c, 3g, and 3h), while two (rcHVT-*SfiI*#1a and #3b) demonstrated green fluorescence in all infected cells and the other three (rcHVT-*SfiI*#3d, 3e, and 3f) were partially fluorescent (Fig. 3-3, Table 3-2).

To identify BAC excision, the reconstituted viruses were serially passaged. Green fluorescence disappeared in the three clones with partial eGFP expression after the first passage (rcHVT-*SfiI*#3d, 3e, and 3f; Table 3-2), and in rcHVT-*SfiI* #3b clone, it decreased after 10 passages, but was still observed in all rcHVT-*SfiI*#1a plaques after multiple passages. PCR analysis of this clone at the 2nd passage demonstrated amplification of eGFP and mini-F sequences (Region 1; Figs. 3-4A and 4B(a)) and the bridge between the chloramphenicol resistance cassette and HVT (Region 2; Figs. 3-4A and 4B(b)), indicating the presence of the inserted sequences. rcHVT-*SfiI*#3b and 3f also retained eGFP, chloramphenicol resistance cassette, and the mini-F (Figs. 3-4A and 4B(a, b)). Then, the insertion site region of the FC126 genome (Region 3; Fig. 3-4A) was analyzed by PCR, which was supposed to amplify a 2.0-kbp fragment

if the viral genome did not carry eGFP, chloramphenicol resistance cassette, and the mini-F in the TK region. In addition, the amplified fragment (Region 3) should be digested by *Sfi*I into 1.6-kbp and 400-bp fragments if recombination occurred between the virus genome and pUC18-HVT-TK-*Sfi*I, since there was no *Sfi*I site in the TK region of parent FC126, while pHVT-BAC should be digested by *Sfi*I into 8.0-kbp, 1.6-kbp, and 500-bp fragments (Fig. 3-4A). The results showed that rcHVT-*Sfi*I#3a and 3h were not digested with *Sfi*I and rcHVT-*Sfi*I#1a did not show the 2.0-kbp band (Fig. 3-4B(c)), which is consistent with its eGFP expression in CEFs. Other clones showed 2.0-kbp, 1.6-kbp, and 400-bp bands. Sequencing of the 2.0-kbp fragments revealed that rcHVT-*Sfi*I#3a and 3h did not have the *Sfi*I recognition site in the TK region and carried sequences homologous to the intact TK region, while rcHVT-*Sfi*I#3b, 3c, 3d, 3e, 3f, and 3g had the *Sfi*I site.

Growth kinetics and plaque morphology of the reconstituted viruses. To analyze characteristics of the reconstituted viruses, pHVT-BAC #1 and #3 were digested with I-*Sce*I, and transferred to CEFs together with pUC18-HVT-TK. Plaques with and without green fluorescence (rcHVT-BAC(+) and rcHVT-BAC(-), respectively) were obtained from both #1 and #3 clones, and the respective viruses

were isolated (rcHVT#1-BAC(+), rcHVT#3-BAC(+), rcHVT#1-BAC(-), and rcHVT#3-BAC(-)). Growth kinetics and plaque morphology of reconstituted viruses were compared with those of the parental FC126 strain (Fig. 3-5). While non-fluorescent clones showed growth almost similar to that of the FC126 strain, significant differences were observed between the parental FC126 strain and rcHVT#1-BAC(-) at 48 hr, and between FC126 and rcHVT#3-BAC(-) at 24 and 48 hr after infection (Fig. 3-5A). In contrast, the titers of fluorescent viruses were lower than those of the parental strain, and significant differences were observed between the FC126 strain and fluorescent viruses at 24, 48, and 72 hr after infection. Similar results were obtained for plaque morphology: there was no significant difference between plaque sizes of non-fluorescent clones rcHVT #1-BAC(-) and rcHVT#3-BAC(-) and the FC126 strain, while the average plaque size of fluorescent viruses was significantly smaller (Fig. 3-5B).

DISCUSSION

In this chapter, I demonstrated a new strategy to excise BAC cassette from the viral BAC vector by cotransfecting eukaryotic cells with a linearized virus-BAC construct and a homologous plasmid. Traditional methods have certain inherent problems, such as leaving scars of one *loxP* or *FRT* sequence, laborious purification, complicated construction of virus BAC, or instability of the construct (1)(6)(9)(47)(59). My strategy termed RIBON (Removal of Inserted BAC after linearizatiON) is simple and does not need laborious purification steps: it only requires transfection of eukaryotic cells with the linearized virus BAC and homology plasmid. Furthermore, it does not involve the generation of complex virus BAC structures, and leaves no scars of the BAC sequence in the reconstituted virus. Moreover, by inserting *I-SceI* site adjacent to the mini-F sequences in *E. coli* using the Red-Recombinase technology, this method can be easily applied to the cases when the virus BAC has been constructed with the aim to be used with other BAC-excision methods. Combined with two-step selection procedures, such as *galK* positive/negative selection for gene insertion or modification (61), the RIBON strategy enables to generate viruses without scars. In addition, the RIBON strategy can be applied with “en passant” mutagenesis (57) to modify or insert genes, if other homing enzymes, such as *I-CeuI*, *PI-PspI*, and *PI-*

SceI are used for linearization.

After transfection of CEFs, some clones still retained eGFP fluorescence, possibly because of transient expression of linearized but not recombined pHVT-BAC or traces of circular I-*SceI*-undigested pHVT-BAC. Reconstituted HVT-*SfiI*#1a was probably generated from circular pHVT-BAC, as indicated by the retention of eGFP expression after 10 rounds of passaging and the presence of BAC sequence confirmed by PCR. On the other hand, in rcHVT-BAC-*SfiI* clones #3b, 3d, 3e, and 3f, which lost eGFP expression after several passages, linearized but not recombined or circular pHVT-BAC DNA may coexist with the correctly recombined virus. As growth of the BAC-carrying virus is slower than that of the BAC-free virus, the latter would be selected in culture after several passages if these viruses are co-cultured. Therefore, it should be useful in the RIBON strategy to insert the BAC cassette into the region important for viral replication, so that BAC-carrying viruses would undergo negative selection if co-cultured with BAC-excised viruses.

PCR analysis revealed that the lack of eGFP expression may not indicate the loss of BAC sequence, since not only rcHVT-BAC-*SfiI*#1a and 3b expressing eGFP, but also eGFP-negative 3f retained BAC DNA at the second passage. However, no BAC sequence was detected in any clone except rcHVT-*SfiI*#1a at the 10th passage (data

not shown), and the disappearance of eGFP expression corresponded to the absence of BAC-carrying viruses. Thus, while viruses with partial green fluorescence probably lost BAC DNA after several passages, it will be safer to choose the clones without fluorescence after transfection.

Sequencing of PCR amplicons revealed that rcHVT-*Sfi*I#3b, 3c, 3d, 3e, 3f, and 3g had a *Sfi*I recognition site and that they had correctly recombined with pHVT-TK-*Sfi*I. The uncut 2.0-kbp bands were probably a result of incomplete *Sfi*I digestion, since this restriction enzyme requires paired recognition sites for cleavage, and the presence of only one site decreases DNA digestion by *Sfi*I (62). In contrast, rcHVT-*Sfi*I#3a and 3h did not have the *Sfi*I restriction site, despite the presence of the site in both of the original pHVT-BAC and pUC18-HVT-TK-*Sfi*I constructs. The sequences of the two amplicons were homologous to that of the intact TK region lacking the *Sfi*I site, which may be eliminated via recombination between the 50-bp duplication sequences flanking the BAC cassette and *Sfi*I recognition sites during the reaction of linearized pHVT-BAC with pUC18-HVT-TK-*Sfi*I, or during self-recombination of linearized pHVT-BAC.

There is significant difference in growth kinetics between the parental FC126 strain and BAC-positive as well as BAC-negative viruses. The difference between FC126

and BAC-negative viruses may be a result of HVT-BAC construction rather than BAC excision. Thus, genomic mutation in the reconstituted virus-BAC has been reported for Marek's disease virus (33); besides, HVT-BAC is typically derived from a single population of the parental FC126 strain, although FC126 is not a clonal virus. *In vivo* studies of the reconstituted viruses are required, considering that changes in the pathogenic profile were detected for reconstituted Marek's disease viruses (22).

In this chapter, the I-*SceI* recognition site, eGFP, chloramphenicol resistance cassette, mini-F, *loxP*, and 50-bp duplication sequences were inserted into the virus-BAC construct. However, *loxP* and 50-bp duplication sequences were inserted to be used with other BAC excision approaches and were not necessary for the RIBON strategy. Further studies of the RIBON method applied to other herpesviruses without *loxP* and the 50-bp duplication sequence are needed. In these cases, the RIBON strategy would be used with other homing enzyme such as I-*CeuI*, PI-*PspI*, and PI-*SceI*. Furthermore, this novel strategy may be also applied for gene insertion, if an appropriate transfer plasmid carrying the gene flanked with sequences homologous to the insertion region of the BAC cassette is used. Thus, the RIBON strategy should be a useful method for the excision of the BAC cassette and gene insertion.

Table 3-1. Primers used for generating plasmids and *in vitro* stability analysis

Primer	Sequence (5'-3')
F-HVT- <i>Sall</i> -45700	GCGTCGACTTGTCGGGGTGGCCA
R-HVT- <i>SacI</i> -48967	GCGAGCTCTCCAAAGGTCTGAGTCTGC
R-HVT- <i>SfiI</i> -47316	TAGGCCGGGGGGGGCCGGCACCCTGTGG
F-HVT- <i>SfiI</i> -47317	CCGGCCCCCCCCGGCCTATCCAGCATTAAT
F- <i>SfiI</i> - <i>LoxP</i> -CMV	GGCCCCCCCCGGCCATAACTTCGTATAGCATACATTATACGAAGTTATAAGGCTGCAGAGTTATTAATAGTAA
R-eGFP-CMV	GCCCTTGCTCACCATGGATCTGACGGTTCACT
F-CMV-eGFP	AGTGAACCGTCAGATCCATGGTGAGCAAGGGCGAGGAGCT
R- <i>SfiI</i> - <i>LoxP</i> - <i>Sall</i> -eGFP	GGCCGGGGGGGGCCATAACTTCGTATAATGTATGCTATACGAAGTTATGTCGACCCCCAGCTGGTTCTTTCCG
F-TK-duplication	CCGCCACAGGGTGCCTATCCAGCATTAATATAATTGCTGGAGTATCGCATATTTCTATTTTTCC
R-TK- <i>SfiI</i> -I- <i>SceI</i>	TAGGCCGGGGGGGGCCATTACCCTGTTATCCCTAAGGAAAAATAGAAATATGCGATACTCCAGCA
F-eGFP	GTGAGCAAGGGCGAGGAG
R-BAC	GGGTAACGATTATCGCCCAAC
F-cm	GTACTGCGATGAGTGGCAG
R-HVT-47750	CCTCGAAGACAATTGCCAGC
F-HVT-46898	AATGGCCAGGAGAGTTCGG

Table 3-2. eGFP expression by reconstituted HVT-*SfiI*

Clone number	Passage 0	Passage 1	Passage 2	Passage 9	Passage 10
rcHVT-<i>SfiI</i> #1a	+	+	+	+	+
rcHVT-<i>SfiI</i> #3a	-	-	-	-	-
rcHVT-<i>SfiI</i> #3b	+	partial	partial	partial	-
rcHVT-<i>SfiI</i> #3c	-	-	-	-	-
rcHVT-<i>SfiI</i> #3d	partial	-	-	-	-
rcHVT-<i>SfiI</i> #3e	partial	-	-	-	-
rcHVT-<i>SfiI</i> #3f	partial	-	-	-	-
rcHVT-<i>SfiI</i> #3g	-	-	-	-	-
rcHVT-<i>SfiI</i> #3h	-	-	-	-	-

+, eGFP expression in all plaques; partial, eGFP expression in some plaques; -, no eGFP expression

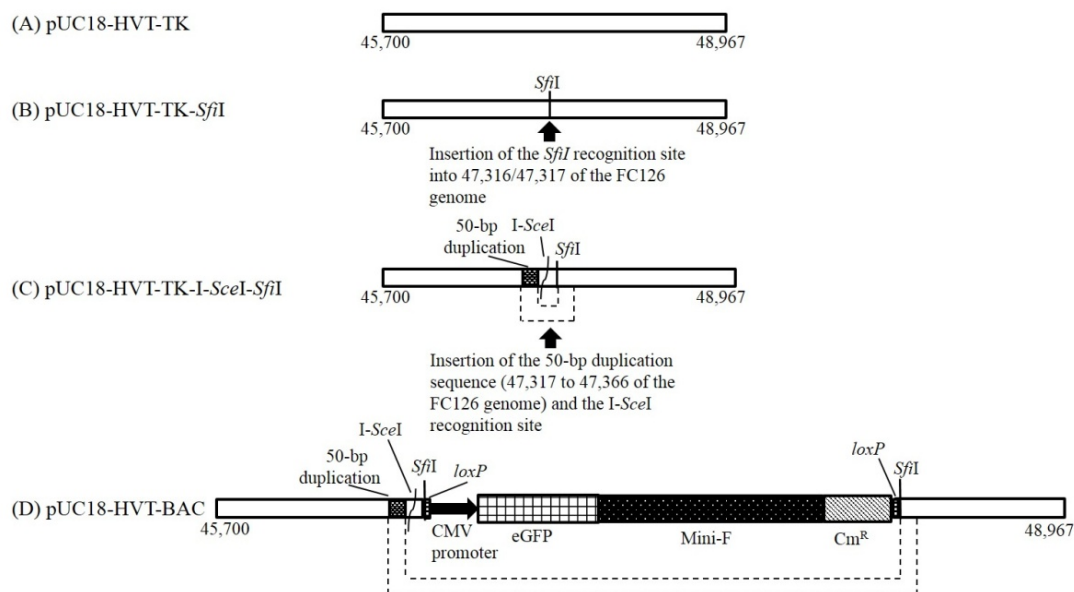


Figure 3-1. Schematic diagrams of the plasmids.

(A) pUC18-HVT-TK. The region from 45,700 to 48,967 nucleotides (nts) of the herpesvirus of turkeys (HVT) FC126 strain genome was cloned into pUC18. (B) pUC18-HVT-TK-*Sfi*I. The *Sfi*I recognition site was introduced between nts 47,316 and 47,317 of the FC126 genome, and the 45,700–48,967 region was cloned into pUC18. (C) pUC18-HVT-TK-I-*Sce*I-*Sfi*I. A 50-bp duplication sequence (nts 47,317–47,366 of the FC126 genome) and the I-*Sce*I recognition site were inserted before the *Sfi*I site. Dashed lines show homologous sequences. (D) pUC18-HVT-BAC. *LoxP*, eGFP, mini-F, and chloramphenicol resistance cassette sequences were inserted into the *Sfi*I site of pUC18-HVT-TK-I-*Sce*I-*Sfi*I. Dashed lines show homologous sequences. Cm^R indicates the chloramphenicol resistance gene.

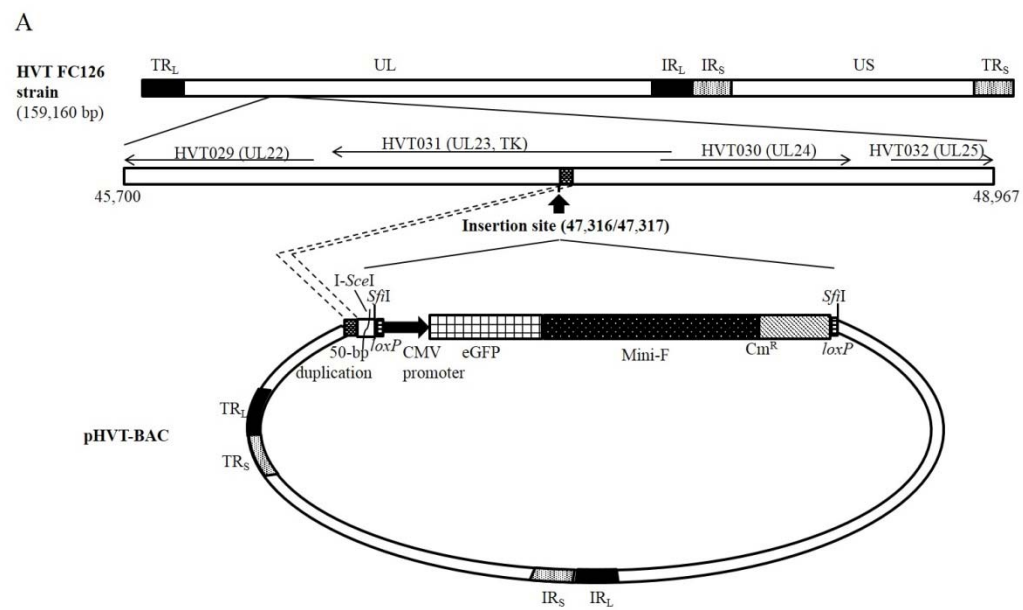


Figure 3-2A

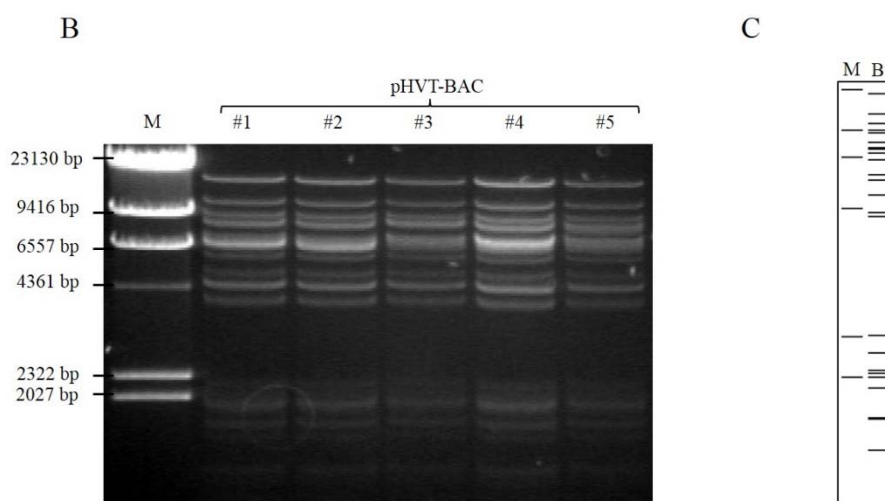


Figure 3-2B and 2C

Figure 3-2. Construction of pHVT-BAC.

(A) A schematic diagram of pHVT-BAC. Duplication sequence of FC126, I-SceI

recognition sequence, *loxP*, eGFP, mini-F, and chloramphenicol resistant cassette were inserted between nts 47,316 and 47,317 of the FC126 genome. Cm^R indicates the chloramphenicol resistance gene. Dashed lines show homologous sequences. (B) *EcoRI* digestion pattern of pHVT-BAC in 0.8% agarose gel. Numbers indicate virus clones. (C) Expected band pattern of pHVT-BAC constructed using the Gene Construction Kit. B indicates pHVT-BAC. M, molecular weight markers.

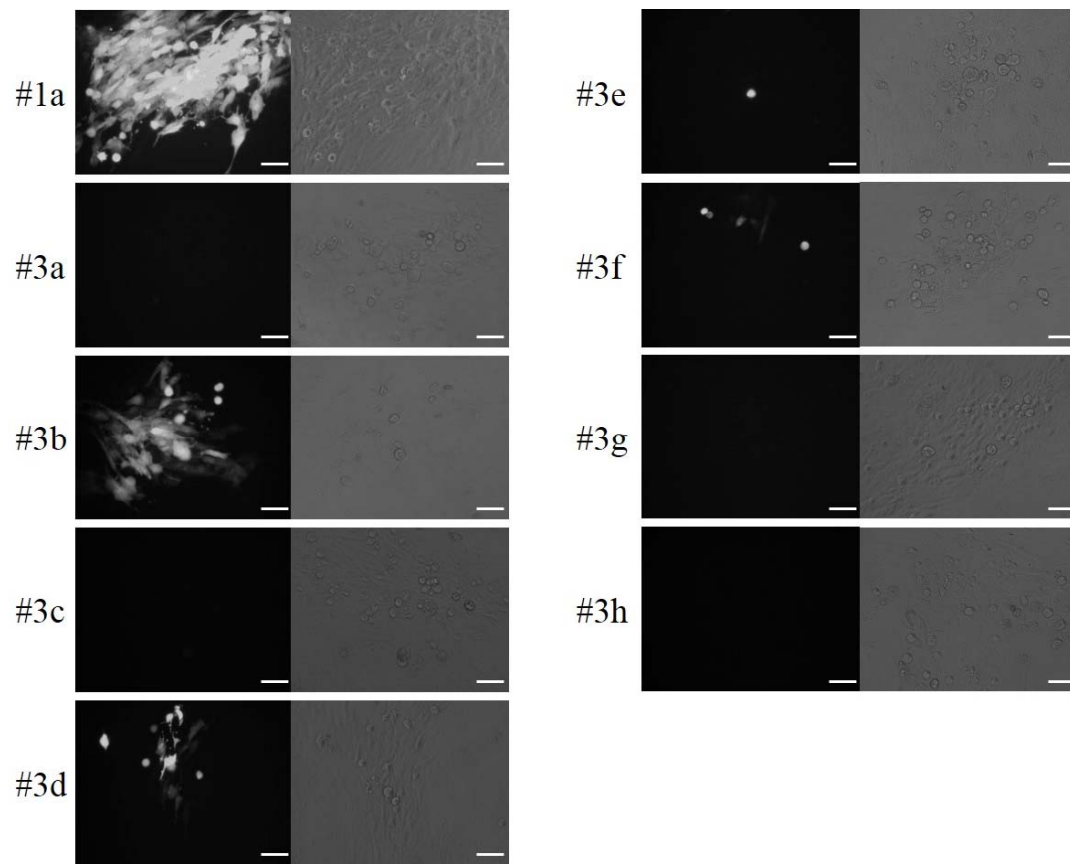


Figure 3-3. Plaques produced by reconstituted HVT-*Sfi*I in chicken embryo fibroblasts.

Cells were transfected with linearized pHVT-BAC and pUC18-HVT-TK-*Sfi*I and analyzed for plaque formation and eGFP expression five days after transfection. Left panels, eGFP fluorescence; right panels, bright field microscopy. Scale bars, 50mm.

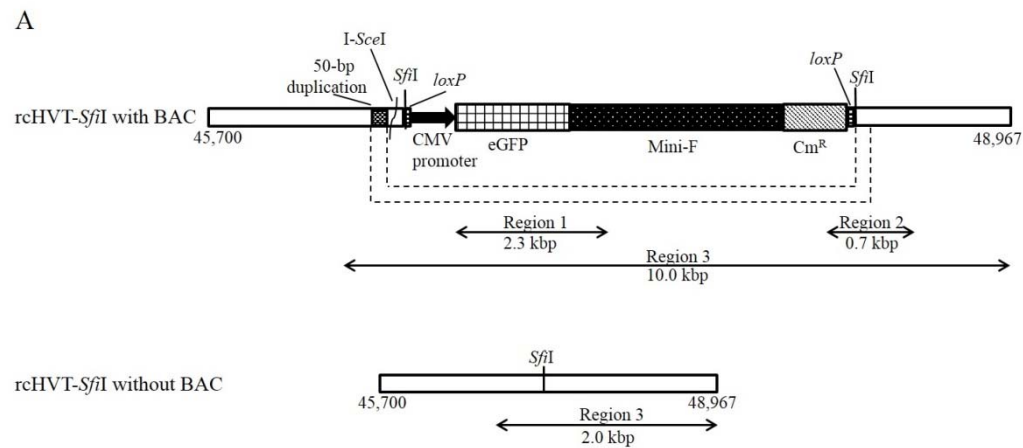


Figure 3-4A

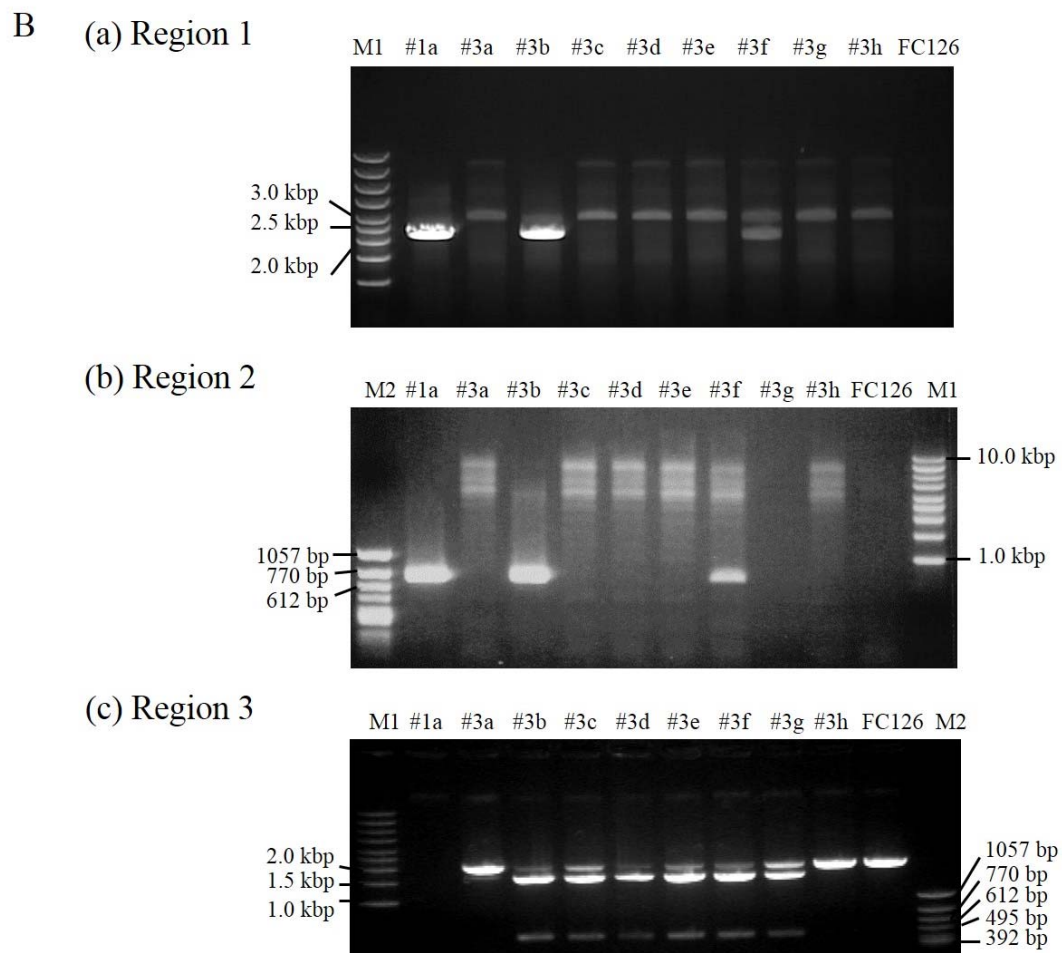


Figure 3-4B

Figure 3-4. PCR analysis of rcHVT-*Sfi*I.

(A) Schematic diagram of the amplified region of rcHVT-*Sfi*I with and without BAC. The size of the region is indicated below the arrow. Dashed lines show homologous sequences.

(B) PCR analysis of rcHVT-*Sfi*I. DNA extracted from chicken embryo fibroblasts infected with rcHVT-*Sfi*I at the 2nd passage or FC126 DNA was used as templates. (a) A region between the eGFP-encoding gene and mini-F sequences (Region 1). (b) A region between chloramphenicol resistance cassette and the insertion site of the FC126 genome (Region 2). (c) The insertion site region of the FC126 genome (Region 3). PCR-amplified Region 3 fragments were digested with *Sfi*I and analyzed by agarose gel electrophoresis. M1 and M2, molecular weight markers.

A

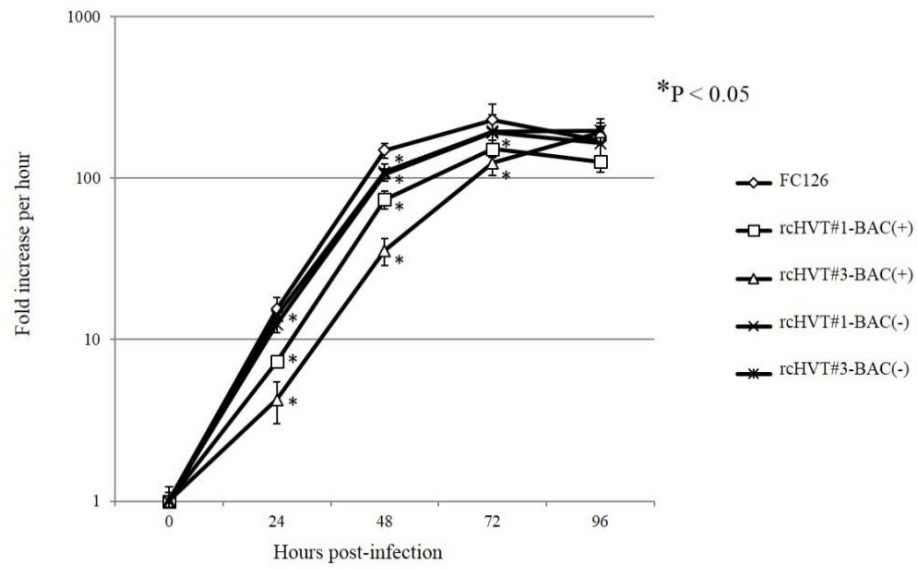


Figure 3-5A

B

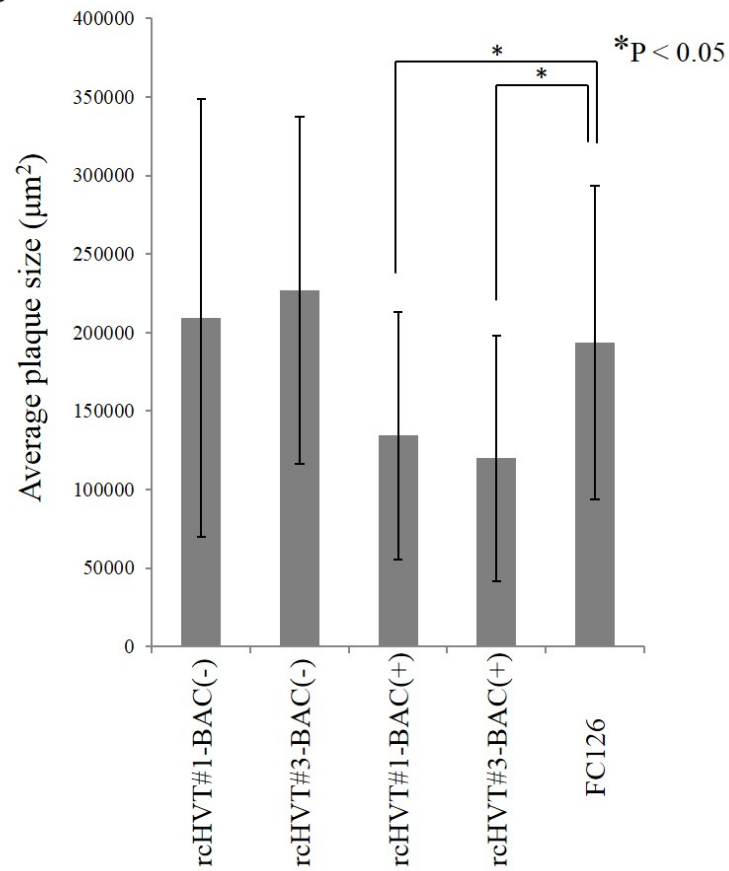


Figure 3-5B

Fig. 3-5. Growth characteristics of rcHVT.

(A) Growth kinetics of rcHVT and parental FC126. Chicken embryo fibroblasts were infected with rcHVT-BAC or FC126 at the MOI of 0.001 and analyzed at the indicated times post-infection for viral titers by the plaque assay. * $P < 0.05$ was considered statistically significant. (B) The average plaque size produced by rcHVT and FC126. Cells were infected as above for four days, and analyzed for plaque size by immunocytochemistry. The data are expressed as the mean \pm SD (n = 50 plaques); * $P < 0.05$ was considered statistically significant.

GENERAL CONCLUSION

MD is one of the most important diseases in the poultry industry. Many vaccines have been developed against MD. Because of its ability for persistent infection, capacity for accommodation of foreign genomes, and safety, HVT is used not only as a live attenuated vaccine, but also as a vaccine vector. Although the efficacy of HVT vectors has been well established through multiple studies, there are a number of disadvantages in using HVT and HVT vectors. These include low efficacy against very virulent MDV strains and low compatibility of two HVT vector vaccines. The author demonstrated a new vaccination strategy for the poultry industry that utilizes a combination of MDV and HVT vector vaccines.

In Chapter 1, the author described the construction of Rispens vector vaccines. Rispens vector vaccines have been extensively studied by several groups since the 1990s (48). However, there are no commercially available Rispens vector vaccines despite the superior immunogenicity reported in these studies. In this chapter, the author aimed to construct a Rispens vector vaccine that can be used commercially. Rispens/IBD viruses driven by different promoters were constructed and their protective efficacy against IBD was investigated. Among these viruses, Rispens/Coa5-IBD showed excellent protective

efficacy against virulent IBD in both SPF and commercial chickens. It should be noted that this is the first report of a Rispons/IBD vaccine that can induce good immunity against virulent IBD in both SPF and commercial chickens. Thus, Rispons/Coa5-IBD can be used for commercial application. In addition, the author constructed a Rispons vector system that could potentially help the poultry industry like the HVT vector system.

In Chapter 2, mixed inoculation of HVT vector vaccine and Rispons vector vaccine in chickens was studied. HVT is often used in combination with Rispons to enhance the efficacy against very virulent MDV strains and the safety of the mixed inoculum of HVT and Rispons is well known. However, there are no reports of mixed inoculation studies of HVT vector vaccine and Rispons vector vaccine. In this chapter, the author inoculated HVT/ND and Rispons/IBD vector vaccines simultaneously into chickens. Chickens administered with these mixed inoculations showed no interference on the protective efficacy against virulent NDV and IBD. They also displayed protection against virulent MDV strains. In addition, no recombination between Rispons/IBD and HVT/ND was observed. Thus, mixed inoculation provides protection against MDV, IBD, and NDV with a single administration. This could be a remarkable finding for the poultry industry because it can significantly cut the cost of vaccination. Further studies will be needed to confirm long-lasting immunity.

In chapter 3, the author investigated a new strategy to excise BAC-derived sequences from viruses. Although the BAC technology is useful for the manipulation of herpesvirus genomes, BAC-derived sequences are undesirable in vaccines. Therefore, it is important to remove all BAC-derived sequences from vaccines before commercial application. In chapter 1, the author employed Cre/*LoxP* method to excise BAC-derived sequences. However, this strategy left a scar of the *LoxP* sequences in the rescued Rispens-IBD virus. Cottingham *et al.* reported a method for generating a markerless virus (9). However, this method could not be applied to the Rispens/IBD in Chapter 1 because it requires complicated virus BAC construction initially; it is difficult to use this method once virus BAC has been constructed. Therefore, the author developed a new strategy to excise BAC-derived sequence without scarring that can be used even after the virus BAC has been constructed with other BAC-excision methods. The study was performed with HVT because the study on HVT vector and HVT-BAC was a step ahead of the study on the Rispens vector. Some reconstituted HVTs were successfully freed of BAC-derived sequences using the RIBON method. This strategy could allow the production of markerless recombinant viruses in Rispens vectors as well. Further studies on the RIBON strategy with Rispens vectors will be needed before it will be ready for commercial application.

In conclusion, the author successfully established Rispons vector vaccine for commercial use. This study will be of great potential help to the poultry industry for the development of Rispons vector vaccines and virus-BAC usage.

ACKNOWLEDGMENTS

I would like to show my greatest appreciation to everyone who has contributed to this thesis, in particular to:

CEVA-JAPAN K.K. for supporting my study and publications.

Dr. Motoyuki Esaki (CEVA-JAPAN K.K.) for indispensable support, help, advice, and all his kindness to complete studies.

Mr. Shuji Saito, Dr. Takanori Sato, and Dr. Atsushi Yasuda for great support, help, and advice.

I also owe my deepest gratitude to:

Associate Professor Kentaro Kato (Obihiro University of Agriculture and Veterinary Medicine) and Professor Taisuke Horimoto (The University of Tokyo) for giving me a chance to take Ph.D. and for teaching me the basic of microbiology.

Mr. Keita Sugiura (Kaken Pharmaceutical Co., Ltd) for teaching me the basic of experiment and his kindness.

Ms. Kyoko Kano (The University of Tokyo) for assisting me and her indispensable kindness.

Professor Hiroomi Akashi (The University of Tokyo) for giving me a chance to work as a researcher and for teaching me the basic of microbiology.

Finally, I am also grateful to my husband Dr. Kohei Saeki for his outstanding supports, affections, and encouragement.

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